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Influence of Stress Treatments on the Resistance of *Lactococcus lactis* to Freezing and Freeze-Drying

Chan Lin
Utah State University

INFLUENCE OF STRESS TREATMENTS ON THE RESISTANCE OF *LACTOCOCCUS LACTIS* TO FREEZING AND FREEZE-DRYING

by

Chan Lin

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ABSTRACT

Influence of Stress Treatments on the Resistance of *Lactococcus lactis* to Freezing and Freeze-Drying

by

Chan Lin, Master of Science

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Major Professor: Dr. Jeffery R. Broadbent
Department: Nutrition and Food Sciences

This study investigated the effect of cold, heat, or osmotic shock treatment on the resistance of *L. lactis* subsp. *cremoris* MM160 and MM310 and *Lactococcus lactis* subsp. *lactis* MM210 and FG2 cheese starter bacteria to freezing and freeze-drying. The ability to withstand freezing at -60°C for 24 h was variable among lactococci, but resistance to this treatment was significantly improved ($P < 0.05$) in most strains by a 2-h cold shock at 10°C or a 25-min heat shock at 39°C (*L. lactis* subsp. *cremoris*) or 42°C (*L. lactis* subsp. *lactis*). Stress treatments that improved lactococcal freeze resistance were also found to significantly ($P < 0.05$) enhance the resistance of most strains to lyophilization. Increased resistance to freezing or lyophilization was not detected when stress treatments were performed in broth that contained erythromycin, which indicated stress-inducible proteins were involved in cell protection. Membrane fatty acid analysis of stress-treated cells suggested that

enhanced resistance to freezing and lyophilization may be related to heat or cold shock-induced changes in cell membrane composition. Heat-shocked cells had a higher 19:0 cyclopropane fatty acid content than did control cells, and cold-shocked cells contained a lower ratio of saturated to unsaturated fatty acids. Other factors must also be involved in cell protection, however, because similar changes in membrane composition were also detected in strains whose resistance to freezing and lyophilization was not improved by heat or cold shock.

(86 pages)

To Youhao

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INTRODUCTION

Lactococcus lactis subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* are important starter bacteria for the production of Cheddar cheese and other fermented dairy foods (34). These bacteria are usually produced as frozen-cell concentrates that are well suited to commercial applications; their quality is uniform; they are easily adapted to different production schedules; and starter activity (i.e., acid producing activity, proteolytic activity) is high and can be tested prior to use. Frozen culture concentrates must be kept at very low temperature (-40°C to -20°C), however, so production, shipping, and storage costs for these bacteria contribute heavily to production overhead (40, 54). Because of these costs, the dairy industry has been interested in the development of lyophilized starter preparations for several years. Unlike frozen cells, lyophilized cultures can be stored for short periods under refrigeration (4°C), yet still possess all of the commercial advantages offered by frozen cell concentrates (8, 54). Unfortunately, relatively few *L. lactis* dairy starter bacteria exhibit the necessary survival and recovery rates from freeze-drying that are required for commercial cheese manufacture (8). This limitation is compounded by the fact that *L. lactis* subsp. *cremoris*, which are preferred over *L. lactis* subsp. *lactis* for cheesemaking (36), generally do not withstand freeze-drying as well as the latter subspecies (8). For this reason, efforts to capitalize on lyophilized starter technology will require new

research to improve the survival and activity of *L. lactis* subsp. *cremoris* starters during lyophilization (8).

In recent years, investigators have realized that bacteria show remarkable resilience and adaptability in response to environmental stresses, and that survival under adverse conditions is frequently enhanced by the expression of an adaptive stress response (for reviews see 7, 11, 17, 19, 60). The most thoroughly characterized stress response in bacteria is heat shock (HS; 19) which, like other stress responses, is characterized by the transient induction of specific proteins and physiological changes which somehow enhance an organism's ability to withstand adverse environmental conditions (19). In addition, stress responses induced under one set of conditions often provide the cell with cross-protection against other hostile yet seemingly unrelated environments (20, 25, 39, 56, 65 For example, microbial resistance to freezing or drying may be enhanced by deliberate induction of a HS, cold shock (CS), or osmotic shock (OS) response (29, 32, 67).

Since freezing and drying each subject cells to a low water stress (24, 59), stress adaptations that improve microbial freeze or desiccation resistance may also enhance survival during lyophilization. Previous work has shown that *L. lactis* has active HS, CS, and OS responses (44, 51, 66) and that CS has a cryoprotective effect on *L. lactis* subsp. *lactis* (31, 52). In this study, we investigated the effect of HS, CS, and OS on cryotolerance in commercial *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* starters, and determined whether treatments that conferred enhanced freeze resistance also helped to

promote cell viability during lyophilization. Results showed that freeze resistance was variable among lactococci, but could be improved in most strains by CS or HS treatments. Stress treatments which improved lactococcal freeze resistance also enhanced the resistance of most strains to lyophilization. Increased resistance to freezing and lyophilization was not detected when stress treatments were performed in the presence of erythromycin, which indicated that stress-inducible proteins were required for cell protection. Membrane fatty acid analysis suggested that enhanced resistance to freezing and lyophilization may be related to stress-induced changes in cell membrane composition. Heat-shocked cells had a higher 19:0 cyclopropane acid content than did control cells, and membranes of cold-shocked cells contained a lower ratio of saturated to unsaturated fatty acids. Other factors must also be involved in cell protection, however, because similar changes were detected in strains whose resistance to freezing and lyophilization was not improved by cold or heat shock.

LITERATURE REVIEW

Lactococcus lactis subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* are utilized worldwide as starter bacteria for the manufacture of cheese and fermented milks (34). In Cheddar cheese, *L. lactis* subsp. *cremoris* starters are preferred over *L. lactis* subsp. *lactis* because the former subspecies is thought to promote superior cheese flavor development (36). This is because *L. lactis* subsp. *cremoris* are more sensitive to environmental stress (e.g., less tolerant to temperature extremes and high salt levels) than *L. lactis* subsp. *lactis*, and it is easier to control acid production with the former subspecies during cheese production. Improved control over the starter acidification translates into better control over the fermentation, which in turn facilitates the production of more uniform, high-quality cheese (23).

Starter production. Historically, *L. lactis* starter cultures were distributed to cheese manufacturers in liquid form, but this practice had several disadvantageous consequences. First, days or even weeks of successive subculturing were required to obtain sufficient inoculum for large cheese vats, and cultures were quite vulnerable to bacteriophage attack during this period (40). In addition, successful manipulation of the starter required employees with microbiological training, so the process was both laborious and expensive.

Advances in biomass production technology led to the development of more convenient forms of starter cultures, such as frozen-cell concentrates,

that offer significant savings in labor and material costs (8, 54). These starters are easy to use; their quality is uniform; they are easily adapted to different production schedules; and starter activity (i.e., acid producing activity, proteolytic activity) is high and can be tested prior to use. Frozen culture concentrates must be kept at very low temperature (-40°C to -20°C), however, so production, shipping, and storage costs for these bacteria contribute heavily to production overhead (40, 54). Because of these costs, the dairy industry has been interested in the development of lyophilized starter preparations for several years.

Lyophilization. Lyophilization, or freeze-drying, involves the removal of water and other solvents from already frozen cells through sublimation (59). In theory, lyophilized cultures should emerge as low-volume powders that can be stored for short periods under refrigeration (4°C) and possess all of the commercial advantages offered by frozen cell concentrates (8, 54). When a suspension of living cells is frozen and then dried, however, several events occur that have detrimental effects on cell viability. As cells are frozen, ice initially forms in the extracellular medium, which results in an increased concentration of solutes outside the cells because of the limitation of liquid water. The difference in osmotic pressure draws water out of the cells so that cells are actually drying as freezing occurs (59). If too much water is removed, cells may accumulate harmful concentrations of intracellular solutes. If too little water is removed, damage occurs as a result of intracellular ice crystal formation (59). Cells also suffer damage during subsequent desiccation steps.

Drying may induce severe damage to proteins, nucleic acids, membranes, and other macromolecules (55).

The cellular damage induced by freezing and drying can be ameliorated to some extent by culture age (late log or stationary phase are best), growth conditions (see below), the lyophilization medium, and the addition of cryoprotectants such as glycerol to that medium (8, 59). Although this information has been used to produce lyophilized lactic starter preparations that are suitable for the inoculation of bulk starter tanks, expanding interest in ready-to-use culture concentrates for the direct inoculation of cheese vats has placed greater emphasis on starter production and preservation methods that promote high cell viability and activity (40, 54, 61). Unfortunately, relatively few lyophilized *L. lactis* starter bacteria exhibit the necessary survival, recovery, and acidification rates that are required for direct inoculation of cheese milk (8). This limitation is compounded by the fact that *L. lactis* subsp. *cremoris*, which are preferred over *L. lactis* subsp. *lactis* for cheesemaking (36), generally do not withstand freeze-drying as well as the latter subspecies (8). As a result, efforts by the dairy industry to capitalize on lyophilization technology require additional research to identify conditions which improve the survival and activity of starter bacteria during lyophilization (8, 29).

Cell membrane lipid composition. As was noted above, one of the primary targets for freeze- or desiccation-induced damage in cells involves the cytoplasmic membrane (14, 55). Under ideal growth conditions, the lipids

in a biological membrane are maintained in a fluid, non-crystalline state wherein the transition temperature (T_m) for the gel-to-liquid-crystal phase is below the environmental temperature (1). During freezing or desiccation, however, the T_m may rise above the ambient temperature, and this situation results in the transient coexistence of gel and liquid-crystal phases. The permeability of this two-phase system is very high, and can be lethal if free water is still available for solute transport (55). Since the correct physical state of the membrane is required for optimal function, maintenance of membrane integrity is a central mechanism in desiccation tolerance (55).

The T_m of a membrane depends on the fatty acid composition of its phospholipids (9), so most bacteria maintain an ideal membrane state by altering fatty acid composition (1). As growth temperature decreases, for example, most cells can lower T_m by incorporating a higher percentage of lower-melting point (e.g., unsaturated) fatty acids into the cytoplasmic membrane (1, 9). Temperature-dependent membrane lipid modification may occur via de novo lipid synthesis or by incorporation of exogenous fatty acids present in the growth medium (9, 48).

The ability of bacteria to modify their membrane fatty acid composition has important practical consequences on the resistance of these organisms to freezing and lyophilization. Work by Goldberg and Eschar (16) showed that growth of *L. lactis* in a medium supplemented with Tween 80 increased the ratio of unsaturated to saturated fatty acids in the cell membrane. This change increased cell membrane fluidity and, as a consequence, improved the

survival of these bacteria during freezing. Subsequent work by Emtseva et al. (14) showed that survival of *Serratia marcescens* and *Erwinia aroideae* during lyophilization was substantially improved when these bacteria were grown in conditions that increased the level of unsaturated fatty acids in their membrane (i.e., incubation at 13° versus 30°C in a medium that contained Tween 80 or oleic acid). These results indicate that efforts to enhance the viability of lyophilized *L. lactis* starters must include treatments that alter membrane lipid composition in a manner that will promote membrane integrity and function in low moisture environments.

Stress adaptation and the heat shock response. Bacteria are highly adaptable organisms and investigators have recently realized that bacterial survival in very harsh environments is frequently linked to the expression of an adaptive stress response (for reviews see 7, 11, 17, 19, 60). The most thoroughly characterized stress response in bacteria and higher cells is heat shock (HS; 19). When cells are briefly exposed to temperatures 5-10°C above the optimal growth temperature, synthesis of many cellular proteins is reduced, but a specific set of proteins, referred to as heat shock proteins (Hsp), is transiently induced. Induction of Hsps is usually followed by an increase in the cells' resistance to high temperature, an effect that is described as induced thermotolerance (19). Although Hsp synthesis has been associated with improved thermotolerance in many organisms, including several species of lactic acid bacteria (2, 4, 6, 15, 19, 58, 65, 66), a direct relationship between Hsps

and cell protection remains controversial because chemical treatments which induce Hsps may not impart thermotolerance (5, 64).

Even though the role of Hsps in thermotolerance is unclear, HS-induced thermotolerance is an established phenomenon, and HS treatment may also induce cross-protection against other environmental extremes such as oxidative or osmotic stress (43, 45, 65). More significantly, experiments by Komatsu et al. (32) demonstrated that HS significantly improved the viability of *Saccharomyces cerevisiae* during freezing and thawing, and showed this effect was dependent on de novo Hsp synthesis. Subsequent work by the same group showed that Hsp synthesis was accompanied by an increase in the unfreezable water content of yeast (33) and an improvement in cell membrane stability against thermal stress (49). Meury and Kohiyama (43) showed that overexpression of the chaperone (and Hsp) DnaK during osmotic challenge in *Escherichia coli* reduced cell lag time by approximately 30% and increased the generation time more than 60%. That result supported the suggestion by Komatsu and co-workers (33) that the cryoprotective effect of Hsps may be related to their function as macromolecular stabilizers (chaperones) because this property is also found in some cryoprotective agents. Macromolecular stabilizers can strengthen the hydrogen-bonded structure of water and, as a consequence, increase the level of unfreezable water that surrounds macromolecules (33). Since enzyme activity requires only a monolayer of water over the protein (55), mechanisms which increase

the unfreezable water content of cells may be very important for cell survival during freezing or drying.

Cold shock. Other stress responses may also help to protect cells against freezing and drying. Brief exposure of microorganisms to suboptimal temperatures, for example, has been shown to induce a cold shock (CS) response that, like HS, is characterized by the transient expression of a set of highly conserved cold shock proteins (Csp) (17). The CS response has been demonstrated in pyschrophilic, mesophilic, and thermophilic prokaryotes (17), and viability studies have shown CS treatment enhanced freeze resistance in many species, including *L. lactis* subsp. *lactis* (17, 31, 52, 63, 67). More detailed investigations of prokaryotic adaptation to low temperature have indicated that Csps have an important regulatory role in this process (17, 67).

Bacterial Csps are defined as the dozen or so molecules that dominate total protein synthesis after a temperature downshift, such that their level relative to the total protein pool is several fold higher in cold-shocked cells than in cells grown at more favorable temperatures (67). In *E. coli* and *Bacillus subtilis*, Csp synthesis is dominated by production of a small (approx. 7.5 kDa), ssDNA-binding protein (CspA and CspB, respectively) that displays greater than 40% identity to the domain of eukaryotic Y-box proteins that also bind single-stranded nucleic acid (DNA or RNA) (17). CspA and CspB are members of a protein family whose homologs have been identified in a wide variety of Gram-negative and Gram-positive bacteria, including *L. lactis* (17,

31). In the presence of RNA polymerase, CspA protein is able to bind at least one CS-inducible gene and this protein (and CspB) also acts as an mRNA chaperone (17, 26). These observations have led to speculation that, like Y-box factors, CspA homologs may be involved in the transcriptional and translational activation of other Csps (17). With respect to cryotolerance, it may be significant to note that two of the other *E. coli* Csps are Hsc66, a homolog of DnaK, and the molecular chaperone termed trigger factor (27, 37). As noted above, previous research has specifically implicated chaperone proteins in cell protection against drying and freezing stress (32, 33, 43). Finally, cold adaptation in bacteria may involve Csp-induced alterations in the cell membrane phospholipid content. Kunklová et al. (35) recently showed that temperature downshift induced intense, de novo membrane phospholipid synthesis in *B. subtilis* that altered the membrane lipid content via the incorporation of more phosphatidylethanolamine (PE) into the cell membrane. While CS did not disturb the synthesis of phosphatidylglycerol (PG), the decreased ratio of PG/PE that resulted was predicted to decrease T_m and, therefore, promote membrane integrity and function at low temperature. More recent research by Nichols and Russell (48) indicates that stress-induced membrane alterations may be due to changes in the utilization of fatty acid primer molecules by the fatty acid synthase system. The observations of Kunklová et al. (35) and Nichols and Russell (48) are particularly important because, as outlined above, the cytoplasmic membrane

is an important target for freeze- or desiccation-induced damage in cells (14, 55). As a result, stress-induced modification of the membrane lipid composition is probably crucial to microbial survival at low temperature.

Osmotic shock. Osmotic shock (OS) is another stress response that has been shown to protect cells in low moisture environments (10). In bacteria, intracellular water is essentially at equilibrium with the extracellular environment because biological membranes are permeable to water, and the cells have a high surface-to-volume ratio (11). Exposure of bacteria to a hypertonic environment induces the expression of osmotic tolerance genes whose products help to protect the cell against dehydration (38). In general, the function of these proteins is to effect an intracellular accumulation of specific solutes, either by transport from the growth medium or de novo synthesis, to concentrations that retard water efflux. Since the molecules involved in this process must not be inhibitory to the cell at high concentrations, they are termed compatible solutes (10). In lactic acid bacteria, prominent compatible solutes include the quarternary amine glycine betaine (betaine), and the amino acids proline and γ-aminobutyric acid (21, 42, 44). Many bacteria are able to synthesize proline and γ-aminobutyric acid from glutamic acid, but betaine must usually be obtained from the growth medium (42, 44). Betaine is a natural component of plant and animal material, however, and lactic acid bacteria are routinely grown on rich media that contain good sources (e.g., yeast extract) of betaine and other compatible solutes (28, 44).

The ability of compatible solutes to protect bacteria against osmotic dehydration suggests they may also promote cellular resistance to conventional drying. This hypothesis is supported by the recent results of Kets et al. (29) who showed that sublethal salt stress treatment induced betaine accumulation and desiccation tolerance in *Lactobacillus plantarum*, *Lactobacillus halotolerans*, and *Enterococcus faecium*. Betaine and proline are known to have an osmoregulatory role in *L. lactis* (44), but their influence on desiccation tolerance has not been reported. Since freezing and drying each subject cells to a low water stress (24), stress adaptations that improve microbial resistance to desiccation or freezing may also enhance survival during lyophilization.

The purpose of this study was to develop methodology and information to test the hypotheses that deliberate induction of HS, CS, or OS responses in *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* would enhance the resistance of these bacteria to freezing and freeze-drying. The specific objectives used to test this hypothesis were:

Objective 1: To evaluate the effect of cold, osmotic, and heat shock treatments on *Lactococcus lactis* culturability after freezing.

Objective 2: To evaluate the effect of stress treatments on culturability of lactococci after lyophilization.

Objective 3: To characterize the influence of effective stress treatments on cell survival, cell growth, acid production, protein synthesis, and membrane lipid composition.

MATERIALS AND METHODS

Bacterial strains. *L. lactis* subsp. *cremoris* MM160, MM310, and *L. lactis* subsp. *lactis* MM210 were acquired from Rhodia, Inc. (Madison, Wis.). *L. lactis* subsp. *lactis* FG2 was obtained from our laboratory culture collection. *L. lactis* FG2 has previously been described as a *L. lactis* subsp. *cremoris* (12), but membrane fatty acid analysis (Analytical Services, Inc., Williston, Vt.) and salt tolerance experiments in our laboratory showed this bacterium was actually a strain of *L. lactis* subsp. *lactis* (unpublished data). All lactococci were propagated at 30°C, stored at 4°C, and maintained by biweekly transfer in M17 broth that contained 0.5% lactose (62).

Prior to use, the species designation for each strain was confirmed by a series of microbiological tests, including Gram stain/morphological determination, assay for catalase production, substrate utilization pattern (Biolog, Inc., Hayward, Calif.), and membrane fatty acid analysis (Analytical Services, Inc.).

Lactococcal freeze tolerance. As noted above, it was the hypothesis of this study that stress treatments which enhanced lactococcal freeze tolerance would also help these bacteria to survive during lyophilization. To investigate this hypothesis, it was first necessary to identify a growth medium in which control cells did not withstand freezing very well. *Lactococcus lactis* subsp. *cremoris* MM160 and *L. lactis* subsp. *lactis* MM210 were selected as model strains to represent each subspecies for this and other experiments in

the study. Cells from a fresh overnight culture (in MEB-Ll or MEB-Lc, Table 1) of each bacterium were inoculated (1%) into 10 ml of fresh broth then incubated at 30°C until the absorbency at 600 nm (A_{600}) = 0.35-0.45 (>10^6 CFU/ml). To reduce the pH effect to the cells, which might occur because of medium acidification during growth (broth pH $\leq$ 6.0 at A_{600} = 0.4), the cells were collected by centrifugation at 3,450 x *g* for 10 min at room temperature, suspended in 10 ml of broth prewarmed to 30°C, and incubated at that temperature for an additional 20 min. Initial cell counts were collected by plating duplicate 0.1-ml samples on Elliker's agar (13), and the cells that remained in a 15-ml plastic centrifuge tube were immediately placed in a test tube rack inside a -60°C freezer for 24 h (67). After the freeze challenge, the cells were thawed quickly in a 30°C water bath to promote culturability (59) and duplicate 0.1-ml samples were plated on Elliker's agar. Cells plated before and after the freeze challenge were enumerated after anaerobic incubation at 30°C for 24 h, and lactococcal freeze resistance was expressed as the fraction of the original cell population that was still able to form colonies (i.e., remained culturable) after freeze challenge.

Stress treatments. Lactococci for HS, CS, and OS treatments were propagated in one of two modified Elliker's broth media (MEB-Ll or MEB-Lc, Table 1). Stress treatments to *L. lactis* subsp. *cremoris* MM160 and MM310 were performed in MEB-Lc, and experiments with *L. lactis* subsp. *lactis* MM210 and FG2 were done in MEB-Ll. The rationale used to select these media is presented in the Results section. Mid-log phase cells for stress

TABLE 1. Composition of the modified Elliker's broth media (MEB) used for stress tolerance studies

Ingredient	Grams per liter	
	MEB-Lc	MEB-Ll
Tryptone	20.0	10.0
Yeast extract	5.0	2.5
Lactose	5.0	2.5
Sodium chloride	4.0	4.0
Sodium acetate	1.5	1.5

treatments were prepared as described for freeze tolerance studies. After the 20-min incubation at 30°C, the cells were collected again and suspended in 10 ml of MEB-Lc or MEB-Ll that had been adjusted to induce a HS, CS, or OS response. Lactococci were heat shocked by suspension in broth that had been prewarmed to 42°C (66) or other HS temperatures (see results) then incubated at the HS temperature for 25 min. Cells for CS treatments were suspended in broth precooled to 10°C (31) and incubated at that temperature for 1, 2, 3, or 4 h. Osmotic shock was induced in *L. lactis* subsp. *cremoris* strains by suspending the cell pellet in MEB-Lc broth that contained 1.75, 2.25, or 2.75% NaCl, whereas *L. lactis* subsp. *lactis* MM210 and FG2 were salt shocked in MEB-Ll broth with 4.5, 5.0, or 5.5% NaCl. The rationale used to select these salt concentrations is outlined in the Results section. Lactococci in salt solutions were incubated for 30 min at 30°C (44, 65), collected by

centrifugation, and suspended in an equivalent volume of fresh MEB-Lc or MEB-Ll that did not contain salt. Duplicate 0.1-ml samples were plated on Elliker's agar before and after each stress treatment, and colonies were enumerated after anaerobic incubation at 30°C for 24 h.

Effect of stress treatments on lactococcal freeze tolerance. The influence of individual HS, CS, or OS treatments on lactococcal freeze tolerance was investigated using several 10-ml mid-log phase cell suspensions prepared as described for freeze tolerance studies. After the 20 min incubation at 30°C, duplicate 0.1-ml samples from each tube were plated on Elliker's agar to obtain an initial cell count. Cells in one 15-ml centrifuge tube (control) were immediately placed on a test tube rack inside a -60°C freezer, and lactococci in one or more additional tubes were collected by centrifugation at 3,450 x *g* for 10 min at room temperature, then heat, cold, or osmotic shocked as described above. When a stress treatment was completed, duplicate 0.1-ml samples from that tube were plated on Elliker's agar, and the remaining cells were placed at -60°C for 24 h. After the freeze challenge, the cells were thawed quickly in a 30°C water bath, and duplicate 0.1-ml samples were plated on Elliker's agar. Cells plated before and after stress treatments and after the freeze challenge were enumerated after anaerobic incubation at 30°C for 24 h. Lactococcal freeze resistance was measured by dividing the original (pre-stress treated or untreated) cell population with the one obtained after freeze challenge. Values reported in the study represent the mean from at least three replicate experiments.

To determine whether HS, CS, or OS treatments had a cryoprotective effect, the mean fraction of freeze-tolerant cells recovered for each treatment was compared to the mean for untreated control cells using a two-sample *t* test with $\alpha = 0.05$ (46). When a significant effect was detected ($P < 0.05$), the entire experiment was repeated in triplicate to include cells that had been stress treated in broth that contained 5 μg per ml of the protein synthesis inhibitor erythromycin. The purpose of the latter experiment was to determine whether the expression of stress-inducible proteins was required for cell protection.

Effect of stress treatments on lactococcal resistance to lyophilization. Stress treatments that significantly ($P < 0.05$) improved cell survival during freezing were tested for a similar effect against lyophilization. Cells were stress treated as described above, frozen overnight at -60°C in 15 ml centrifugation tubes, and lyophilized in a VirTis (The VirTis Co., Gardiner, N.Y.) 5-liter capacity benchtop freeze dryer for approximately 45 h. The level of residual moisture in lyophilized samples was determined to be 4-5% by the Karl Fischer method (53). After lyophilization, cells were stored overnight at 4°C, rehydrated with 5 ml of MEB-Lc or MEB-Ll broth, and duplicate 0.1-ml samples were collected from each tube and plated on Elliker's agar. The plates were incubated anaerobically at 30°C for 24 h; then, lactococcal resistance to lyophilization was evaluated by dividing the original (pre-stress treated or untreated) cell population with the one obtained after rehydration. The values reported in the study represent the mean cell fraction recovered from

at least three replicate experiments. To determine whether stress treatments enhanced lactococcal resistance to lyophilization, the mean fraction of culturable cells recovered for each treatment was compared to the mean for untreated control cells using a two-sample *t* test ($\alpha = 0.05$). When a significant effect was detected ($P < 0.05$), the experiment was repeated three separate times to include cells that had been stress treated in broth that contained 5 μg per ml of erythromycin.

Lactococcal growth and activity after lyophilization. To investigate the effect of stress treatments on the growth of rehydrated lactococci, 0.1 ml of each cell suspension was transferred to 10 ml of MEB-Lc or MEB-Ll immediately after rehydration, and growth was monitored spectrophotometrically at A_{600} (Turner model 690 spectrophotometer, Mountain View, Calif.) . A sample of the freshly rehydrated cell suspension was also used to monitor the rate of acid production in control and stress-treated cells. These experiments were performed by automated colorimetry (Lab SMART system, LLC, Logan, Utah) (57) using an acid indicator medium recommended by the equipment supplier (Table 2). An aliquot (0.1 ml) of the rehydrated cell suspension was transferred to 10 ml of the acid indicator medium and the change in b* (color shift from blue to yellow) was monitored overnight at 30°C.

Cell membrane lipid composition. Because stress-induced modification of cell membrane lipids may be crucial for bacterial survival in low-moisture environments, experiments were performed to investigate the

TABLE 2. pH indicator medium used to follow acid production by rehydrated cells

Ingredient	Grams or ml per liter
Tryptone	5.0
Yeast extract	2.5
Lactose	1.0
Sterile milk[a]	100 ml
Bromcresol purple	0.04

[a]Ultra-high temperature treated skim milk (Gossner Foods, Inc., Logan, Utah).

influence of cryoprotective stress treatments on the lipid composition of the lactococcal cell membrane. Samples of untreated (control), cold-shocked (2 h at 10°C), and heat-shocked cells were prepared and lyophilized as described above and submitted for membrane fatty acid analysis by gas-liquid chromatography (Analytical Services, Inc., Williston, Vt.). Lyophilized samples of uninoculated MEB-Lc and MEB-Ll media were also included as negative controls. The mass percent values for lactococcal membrane lipids that are reported in the study represent the mean from two separate analyses.

In vivo pulse-labeling of lactococcal proteins. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was used to investigate protein synthesis in *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* during HS and CS treatments. Mid-log phase lactococci were prepared and heat or cold

shocked as described above, except that 100 μCi each of L-[^{35}S]Met and L-[^{35}S]Cys (specific activity >1100 Ci/mM) was added to the cell suspensions 10 min after the stress treatment had been initiated. For CS, label was also added to separate cell suspensions after 40, 100, 160, or 220 min at 10°C to obtain a sequential view of protein synthesis during CS. Once label was added, cells were incubated an additional 20 min at the stress temperature and then radioactive amino acids were chased by addition of 1000-fold excess nonradioactive L-Met and L-Cys (Sigma Chemical Co., St. Louis, Mo.). The cells were collected by centrifugation at 16,000 x *g* for 3 min at room temperature, suspended in 150 ml of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), and lysed with 25 mg of glass beads (Sigma Chemical Co.) by high speed vortex for 8 min (66). The supernatant was collected and 2D-PAGE performed by the method of O'Farrel (50) in a Protean II xi multi-cell electrophoresis unit (Bio-Rad Laboratories, Richmond, Calif.). The amount of label incorporated into protein was measured using 5 μl of trichloroacetic acid precipitated extract as described by VanBogelen and Neidhardt (64). The average amount of radioactivity loaded onto control, HS, and CS gels was 4.2 x 10^5, 1.7 x 10^5, and 2.7 x 10^4 cpm, respectively, for *L. lactis* subsp. *cremoris* MM160, and 2.3 x 10^5, 2.1 x 10^5, and 2.6 x 10^4 cpm, respectively, for control and CS *L. lactis* subsp. *lactis* MM210. The amount of protein loaded onto each gel ranged from 1.3 to 3.2 μg.

Isoelectric separation of lactococcal proteins in the first dimension was achieved with Bio-Lite 3/10 carrier ampholytes (Bio-Rad Laboratories). Ten-

percent SDS-polyacrylamide gels used for the second dimension were fixed in 5% methanol and 7% acetic acid, then stained with Coomassie brilliant blue R (Sigma Chemical Co.) as outlined by Ausubel et al. (3). Bio-Rad broad range protein molecular weight standards were included in second dimension gels. SDS gels were dried in BioDesignGelWrap (BioDesign, Inc., Carmel, N.Y.) as directed by the supplier and autoradiography was performed with Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, N.Y.). Film exposure times were 7-10 d for 2D gels from control and heat-shocked cells, and 6-8 weeks for cold-shocked samples.

RESULTS

Freeze tolerance in untreated *L. lactis*. Lactococcal freeze tolerance was investigated using *L. lactis* subsp. *cremoris* MM160 and *L. lactis* subsp. *lactis* MM210 as model strains for each subspecies. The freeze challenge used for these experiments, 24 h at -60°C, was selected because commercial-scale lyophilization of bacteria is initiated by rapid freezing at -60 to -70°C (59). The lactococci were initially grown and frozen in M17 broth, but the fraction of MM160 and MM210 cells that remained culturable after freeze challenge (> 0.87) was too high to facilitate the statistical comparisons that would be required in the study. This result was somewhat surprising because Kim and Dunn (31) recently reported that viability of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains grown and frozen in M17 fell below 50% after 24 h at -20°C. Since freezing at -60°C will generate smaller, less damaging ice crystals (24), however, the discrepancy was probably due to the colder freeze challenge temperature used in this study. Nonetheless, because it was the hypothesis of this study that stress treatments which enhanced lactococcal cryotolerance would also improve the ability of these bacteria to withstand lyophilization, it was necessary to identify a medium in which control cells were susceptible to freeze challenge at -60°C. For this reason, MM160 freeze-tolerance was re-examined in a medium previously developed to detect lactose fermenting ability in lactococci (bromcresol-purple lactose indicator medium) (41). This medium is a derivative of Elliker's broth, and is not as rich in carbohydrate or

protein as M17. Since a pH indicator was not required for this study, bromcresol purple was omitted, and the new broth medium was designated MEB-Lc (Table 1). Freeze tolerance experiments showed that the fraction of culturable *L. lactis* subsp. *cremoris* MM160 cells recovered after 24 h at -60°C was substantially lower for cells grown and frozen in MEB-Lc versus M17 (less than 0.6 and greater than 0.85, respectively). Growth curves showed the bacterium reached the same maximum A_{600} in both media, which indicated that the reduction in MM160 freeze tolerance was not due to nutritional limitation in MEB-Lc (Fig. 1).

Freeze tolerance in *L. lactis* subsp. *lactis* MM210 was also investigated in MEB-Lc, but the culturability of untreated cells after freezing (0.71 ± 0.18) was quite variable and still rather high. As a result, protein and carbohydrate levels in MEB-Lc were reduced by one-half to obtain the medium designated MEB-Ll (Table 1). When grown and frozen in MEB-Ll, the fraction of culturable *L. lactis* subsp. *lactis* MM210 recovered after the freeze challenge (0.66 ± 0.02) was lower and more uniform than in MEB-Lc. Once again, reduced freeze tolerance in MEB-Ll was not due to nutritional deprivation because MM210 reached the same maximum A_{600} value in MEB-Ll or Elliker's broth (Fig. 2). *Lactococcus lactis* subsp. *lactis* MM210 did attain higher A_{600} values in M17 broth (Fig. 2), but this effect was probably due to the strong buffering capacity (and thus delayed acid inhibition of growth) present in that medium (62). It is also possible that differences in the osmolality of each medium may have affected growth, but this property was not investigated

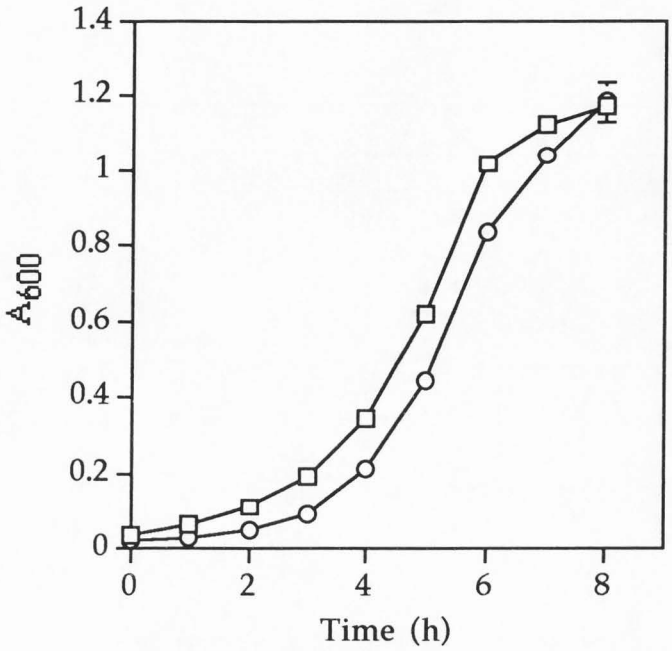


FIG. 1. Growth of *Lactococcus lactis* subsp. *cremoris* MM160 in M17 (□) and MEB-Lc (○) broth media at 30°C.

since control and stress-treated cells would be propagated in the same medium. As mentioned previously, efforts to test the hypothesis that stress treatments which enhanced lactococcal cryotolerance would also improve the ability of these bacteria to withstand lyophilization required the identification of a medium in which control cells were vulnerable to freezing. For this reason, experiments to investigate the influence of stress treatments on the resistance of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* to freezing and lyophilization were performed using cells grown in MEB-Lc and MEB-Ll, respectively.

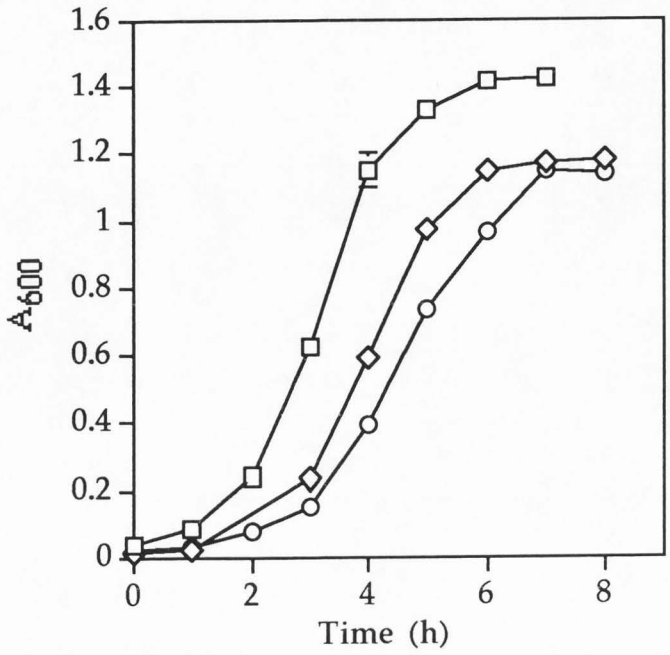


FIG. 2. Growth of *Lactococcus lactis* subsp. *lactis* MM210 in M17 (□), Elliker's (◊), and MEB-Ll (○) broth media at 30°C.

Influence of HS treatment on cryotolerance. Previous studies have indicated that microbial resistance to freezing may be enhanced by deliberate induction of a HS response (32). Data collected in this study showed HS at 42°C for 25 min significantly ($P < 0.02$ and $P < 0.005$, respectively) increased the fraction of *L. lactis* sp. *lactis* MM210 and FG2 cells that remained culturable after freeze challenge (Table 3). Enhanced cryotolerance was not detected in strains MM210 or FG2 when erythromycin was present during HS treatment, which indicated that Hsp expression was essential for cell protection.

TABLE 3. Influence of heat shock (HS) on cell viability and freeze resistance in *Lactococcus lactis* subsp. *lactis* MM210 and FG2

Strain and treatment	Cell fraction recovered[a] After HS	Cell fraction recovered[a] After FC	Adjusted survival[b]	P[c]
MM210:				
None (control)	----	0.40 ± 0.11	----	
HS	0.94 ± 0.13	1.09 ± 0.19	1.17 ± 0.14	< 0.02
HS + Ery[d]	0.95 ± 0.07	0.28 ± 0.06	0.30 ± 0.08	> 0.10
FG2:				
None (control)	----	0.54 ± 0.05	----	
HS	0.98 ± 0.02	1.02 ± 0.06	1.05 ± 0.04	< 0.005
HS + Ery[d]	0.96 ± 0.05	0.56 ± 0.09	0.58 ± 0.09	> 0.25

[a]Cells were heat shocked for 25 min at 42°C. Values represent the mean fraction (± SE) of the initial cell population recovered after HS and after freeze challenge (FC) (24 h at -60°C).
[b]Fraction of the post-HS cell population that was recovered after FC.
[c]Probability values were calculated from a two-sample *t* test comparison between the means from control and HS samples after FC.
[d]HS was performed in broth that contained 5 μg per ml of erythromycin.

In contrast to *L. lactis* subsp. *lactis* MM210 and FG2, HS of *L. lactis* sp. *cremoris* MM160 at 42°C did not significantly increase post-freezing culturability (Table 4). Because *L. lactis* subsp. *cremoris* are more sensitive to high temperature than *L. lactis* subsp. *lactis* (36), experiments to investigate the effect of HS on cryotolerance in MM160 were repeated using lower HS temperatures (Table 4). Those experiments showed HS at 39°C significantly

TABLE 4. Influence of heat shock (HS) temperature on cell viability and freeze resistance in *Lactococcus lactis* subsp. *cremoris* MM160

HS treatment	Cell fraction recovered[a] After HS	Cell fraction recovered[a] After FC	Adjusted survival[b]	*P*
None (control)	----	0.65 ± 0.16	----	
42°C	0.95 ± 0.13	0.77 ± 0.11	0.82 ± 0.16	> 0.15
42°C + Ery[d]	0.77 ± 0.20	0.33 ± 0.11	0.43 ± 0.09	< 0.025
None (control)	----	0.57 ± 0.08	----	
40°C	0.95 ± 0.11	0.71 ± 0.18	0.76 ± 0.24	< 0.10
40°C + Ery[d]	0.63 ± 0.10	0.30 ± 0.12	0.47 ± 0.16	< 0.005
None (control)	----	0.54 ± 0.04	----	
39°C	0.95 ± 0.03	0.76 ± 0.05	0.80 ± 0.04	< 0.02
39°C + Ery[d]	0.77 ± 0.09	0.13 ± 0.04	0.17 ± 0.04	< 0.005
None (control)	----	0.62 ± 0.04	----	
37°C	1.01 ± 0.06	0.69 ± 0.08	0.68 ± 0.05	> 0.10
37°C + Ery[d]	0.94 ± 0.08	0.16 ± 0.04	0.17 ± 0.03	< 0.005

[a]Cells were heat shocked for 25 min. Values represent the mean fraction (± SE) of the initial cell population recovered after HS and after freeze challenge (FC) (24 h at -60°C).
[b]Fraction of the post-HS cell population that was recovered after FC.
[c]Probability values were calculated from a two-sample *t* test comparison between the means from control and HS samples after FC.
[d]HS was performed in broth that contained 5 μg per ml of erythromycin.

enhanced cryotolerance in *L. lactis* sp. *cremoris* MM160 ($P < 0.02$). Efforts to determine whether stress protein synthesis was required for induced cryotolerance in MM160 were inconclusive because erythromycin had a strong deleterious effect on cell recovery (Table 4).

Because HS at 39 and 40°C had the most significant effects on the *L. lactis* subsp. *cremoris* model strain, MM160, the effect of these HS treatments on the freeze resistance of *L. lactis* sp. *cremoris* MM310 was also investigated. As shown in Table 5, those treatments induced a small increase in MM310 cryotolerance, but the effect was not statistically significant ($P > 0.10$).

CS-induced cryotolerance. Cold shock is another stress response that can enhance freeze resistance in many bacteria, including *L. lactis* subsp. *lactis* (31, 52), and studies have suggested that Csps play an important regulatory role in this process (17, 67). Data presented in Table 6 showed that cryotolerance in *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* began to rise after CS at 10°C for 1 h, reached a maximum after 2 to 3 h CS, and then tapered off again. Two-sample *t* tests between the mean fraction of cells recovered after freeze challenge from the untreated control and individual CS samples for each strain confirmed that 2- or 3-h CS treatments significantly ($P < 0.05$) improved lactococcal cryotolerance (Table 6).

Interestingly, *L. lactis* is able to grow at 10°C (47); however, plate counts collected before and after CS treatment revealed that prolonged (3 to 4 h) CS had a deleterious effect on lactococcal culturability (Table 6). Although the fraction of culturable lactococci decreased with prolonged CS, however, two-sample *t* tests between the means from control and adjusted survival (i.e., the fraction of the post-CS population recovered after FC) for CS samples showed that cryotolerance in the post-CS population generally increased with CS time (Table 6). Finally, significant improvements in lactococcal cryotolerance were

TABLE 5. Influence of heat shock (HS) temperature on cell viability and freeze resistance in *Lactococcus lactis* subsp. *cremoris* MM310

Treatment	Cell fraction recovered[a] After HS	After FC	Adjusted survival[b]	P^c
None (control)	----	0.62 ± 0.03	----	
40°C	0.99 ± 0.03	0.68 ± 0.06	0.69 ± 0.05	> 0.10
40°C + Ery[d]	0.76 ± 0.002	0.45 ± 0.16	0.60 ± 0.21	> 0.10
None (control)	----	0.63 ± 0.06	----	
39°C	1.18 ± 0.10	0.72 ± 0.14	0.61 ± 0.08	> 0.25
39°C + Ery[d]	1.17 ± 0.04	0.75 ± 0.08	0.64 ± 0.09	< 0.10

[a]Cells were heat shocked for 25 min. Values represent the mean fraction (± SE) of the initial cell population recovered after HS and after freeze challenge (FC) (24 h at -60°C).
[b]Fraction of the post-HS cell population that was recovered after FC.
[c]Probability values were calculated from a two-sample *t* test comparison between the means from control and HS samples after FC.
[d]HS was performed in broth that contained 5 μg per ml of erythromycin.

not detected when CS was performed in broth that contained erythromycin, which indicated that Csp expression was essential for enhanced freeze resistance.

Influence of OS on lactococcal freeze resistance. Preliminary experiments were performed to identify NaCl concentrations that completely inhibited *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* strains in MEB-Lc and MEB-Ll, respectively (Table 7). From those data, three subinhibitory

TABLE 6. Influence of 10°C cold shock (CS) on cell viability and freeze resistance in *Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis*

Strain and treatment	Cell fraction recovered[a]		Adjusted survival[b]	*P*	
	After CS	After FC		After FC[c]	Adjusted[d]
L. lactis subsp. *cremoris* MM160:					
None (control)	----	0.54 ± 0.21	----		
1 h CS	0.81 ± 0.18	0.60 ± 0.09	0.76 ± 0.10	> 0.25	< 0.10
2 h CS	1.00 ± 0.06	0.90 ± 0.15	0.90 ± 0.13	< 0.01	< 0.01
3 h CS	0.92 ± 0.11	0.89 ± 0.23	0.96 ± 0.17	< 0.10	< 0.05
4 h CS	0.70 ± 0.20	0.72 ± 0.14	1.06 ± 0.22	> 0.10	< 0.05
2 h CS + Ery[e]	1.00 ± 0.04	0.49 ± 0.09	0.49 ± 0.09	> 0.25	> 0.25
L. lactis subsp. *cremoris* MM310:					
None (control)	----	0.61 ± 0.08	----		
1 h CS	0.97 ± 0.14	0.81 ± 0.14	0.83 ± 0.05	< 0.10	< 0.05
2 h CS	1.00 ± 0.09	0.88 ± 0.18	0.87 ± 0.10	< 0.10	< 0.05
3 h CS	1.00 ± 0.06	0.89 ± 0.09	0.89 ± 0.04	< 0.05	< 0.02
4 h CS	0.88 ± 0.17	0.82 ± 0.21	0.92 ± 0.07	> 0.15	< 0.02
2 h CS + Ery[e]	1.00 ± 0.11	0.63 ± 0.07	0.63 ± 0.10	> 0.25	> 0.25

TABLE 6. (continued)

Strain and treatment	Cell fraction recovered[a]		Adjusted survival[b]	*P*	
	After CS	After FC		After FC[c]	Adjusted[d]
L. lactis subsp. *lactis* MM210:					
None (control)	----	0.66 ± 0.03	----		
1 h CS	0.90 ± 0.10	0.71 ± 0.13	0.78 ± 0.13	> 0.25	> 0.10
2 h CS	1.01 ± 0.08	0.96 ± 0.12	0.96 ± 0.14	< 0.02	< 0.05
3 h CS	0.89 ± 0.11	0.83 ± 0.08	0.95 ± 0.17	< 0.05	< 0.05
4 h CS	0.79 ± 0.01	0.74 ± 0.03	0.94 ± 0.04	< 0.05	< 0.01
2 h CS + Ery[e]	0.90 ± 0.05	0.45 ± 0.13	0.50 ± 0.17	< 0.10	> 0.10
L. lactis subsp. *lactis* FG2:					
None (control)	----	0.46 ± 0.11	----		
1 h CS	0.94 ± 0.10	0.70 ± 0.10	0.74 ± 0.04	< 0.005	< 0.001
2 h CS	0.93 ± 0.07	0.73 ± 0.16	0.79 ± 0.18	< 0.01	< 0.005
3 h CS	0.92 ± 0.07	0.77 ± 0.15	0.84 ± 0.15	< 0.005	< 0.0025
4 h CS	0.90 ± 0.12	0.80 ± 0.19	0.89 ± 0.15	< 0.01	< 0.0025
2 h CS + Ery[e]	0.89 ± 0.08	0.44 ± 0.12	0.50 ± 0.14	> 0.25	> 0.25

[a]Values represent the mean fraction (± SE) of the initial cell population recovered after CS and after freeze challenge (FC) (24 h at -60°C).
[b]Fraction of the post-CS cell population that was recovered after FC.
[c]Probability values were calculated from a two-sample *t* test comparison between the means from control and CS samples after FC.
[d]Probability values were calculated from a two-sample *t* test comparison between the means from control and the adjusted survival for CS samples.
[e]CS was performed in broth that contained 5 μg per ml of erythromycin.

TABLE 7. Influence of NaCl concentration on the growth of *Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis*[a]

NaCl concentration (%)	*L. lactis* subsp. *cremoris*		*L. lactis* subsp. *lactis*	
	MM160	MM310	MM210	FG2
0.4[b]	1.10	1.27	1.13	1.19
1.5	0.94	1.02	ND	ND
2.0	0.64	0.79	ND	ND
2.5	0.62	0.74	ND	ND
3.0	0.02	0.01	ND	ND
5.0	ND	ND	0.46	0.49
5.5	ND	ND	0.30	0.24
6.0	ND	ND	0.06	0.06
6.5	ND	ND	0.04	0.07

[a]Values represent the A_{600} attained after 18 h of incubation at 30°C in MEB-Lc (*L. lactis* subsp. *cremoris*) or MEB-Ll (*L. lactis* subsp. *lactis*) . ND, not determined.
[b]NaCl concentration normally present in MEB-Ll and MEB-Lc.

levels of NaCl were selected to study the effect of OS on lactococcal freeze resistance in the two model strains, *L. lactis* subsp. *cremoris* MM160 and *L. lactis* subsp. *lactis* MM210. As shown in Table 8, OS of *L. lactis* subsp. *cremoris* MM160 in MEB-Lc broth that contained 1.75, 2.25, or 2.75% NaCl did not significantly affect the culturability of this bacterium after freeze challenge. In contrast, the cryotolerance of *L. lactis* subsp. *lactis* MM210 was significantly

TABLE 8. Influence of osmotic shock (OS) on cell viability and freeze resistance in *Lactococcus lactis* subsp. *cremoris* MM160 and *L. lactis* subsp. *lactis* MM210

Strain and NaCl concentration (%)	Cell fraction recovered[a]		Adjusted survival[b]	*P*	
	After OS	After FC		After FC[c]	Adjusted[d]
L. lactis subsp. *cremoris* MM160					
0.4 (control)[e]	----	0.57 ± 0.05	----		
1.75	1.15 ± 0.03	0.71 ± 0.05	0.62 ± 0.05	< 0.05	> 0.15
2.25	1.17 ± 0.05	0.73 ± 0.07	0.62 ± 0.03	< 0.05	> 0.10
2.75	0.99 ± 0.05	0.63 ± 0.05	0.63 ± 0.06	> 0.10	> 0.10
2.25 + Ery[f]	0.88 ± 0.09	0.37 ± 0.03	0.43 ± 0.01	< 0.02	< 0.02
L. lactis subsp. *lactis* MM210					
0.4 (control)[e]	----	0.64 ± 0.14	----		
4.5	1.16 ± 0.21	1.11 ± 0.29	1.02 ± 0.46	< 0.05	> 0.10
5.0	1.13 ± 0.12	1.01 ± 0.24	0.89 ± 0.16	< 0.02	< 0.02
5.5	0.87 ± 0.19	0.68 ± 0.08	0.81 ± 0.22	> 0.25	> 0.10
5.0 + Ery[f]	0.89 ± 0.07	0.67 ± 0.15	0.75 ± 0.16	> 0.25	> 0.10

TABLE 8. (continued)

[a]Cells were osmotically shocked for 25 min at 30°C. Values represent the mean fraction (± SE) of the initial cell population recovered after OS treatment and after freeze challenge (FC) (24 h at -60°C).
[b]Fraction of the post-OS cell population that was recovered after FC.
[c]Probability values were calculated from a two-sample *t* test comparison between the means from control and OS samples after FC.
[d]Probability values were calculated from a two-sample *t* test comparison between the means from control and the adjusted survival for OS samples.
[e]NaCl concentration naturally present in MEB-Lc or MEB-Ll.
[f]OS was performed in broth that contained 5 μg per ml of erythromycin.

enhanced ($P < 0.02$) by OS in MEB-LI that contained 5% NaCl (Table 8). Once again, enhanced cryotolerance was not detected when MM210 was osmotically shocked in broth that contained 5% NaCl and erythromycin, which indicated that expression of osmotic shock proteins was required for cell protection. Since OS improved cryotolerance in the *L. lactis* subsp. *lactis* model strain (MM210), the effect of OS in 4.5 and 5.0% NaCl on the freeze resistance of *L. lactis* sp. *lactis* FG2 was also investigated. As shown in Table 9, OS in 5.0% NaCl induced a small increase in FG2 cryotolerance, but the effect was not statistically significant ($P > 0.05$).

Effect of stress treatments on lactococcal survival during lyophilization. Stress treatments that significantly improved cryotolerance in at least two strains of *L. lactis* were tested for a similar effect against lyophilization. As

TABLE 9. Influence of osmotic shock (OS) on cell viability and freeze resistance in *Lactococcus lactis* subsp. *lactis* FG2

NaCl concentration (%)	Cell fraction recovered[a] After OS	After FC	Adjusted survival[b]	P[c]
0.4 (control)[d]	----	0.49 ± 0.15	----	
4.5	0.72 ± 0.14	0.35 ± 0.09	0.48 ± 0.05	> 0.25
5.0	0.69 ± 0.11	0.38 ± 0.08	0.55 ± 0.10	> 0.25

[a]Cells were osmotically shocked for 25 min at 30°C. Values represent the mean fraction (± SE) of the initial cell population recovered after OS treatment and after freeze challenge (FC) (24 h at -60°C).
[b]Fraction of the post-OS cell population that was recovered after FC.
[c]Probability values were calculated from a two-sample *t* test comparison between the means from control and the adjusted survival for OS samples.
[d]NaCl concentration naturally present in MEB-LI.

shown in Table 10, the HS treatments that enhanced freeze resistance in *L. lactis* subsp. *lactis* MM210 and *L. lactis* subsp. *lactis* FG2 also significantly improved the survival of these strains during lyophilization ($P < 0.02$). In agreement with freeze-tolerance experiments (Table 3), Hsp expression appeared to be essential for cell protection because enhanced survival during lyophilization was not detected in MM210 or FG2 when erythromycin was present during HS treatment. Improved resistance to lyophilization was also noted in heat-shocked *L. lactis* subsp. *cremoris* MM160, but the change was not statistically significant ($P > 0.05$).

Data presented in Table 6 showed a 2-h CS at 10°C significantly improved freeze resistance in all of the lactococci used in this study, and this treatment also induced protection against lyophilization in all strains except *L. lactis* subsp. *lactis* FG2 (Table 11). Cold shock treatments in the presence of erythromycin showed protein synthesis during CS was vital to the survival of MM310 and MM210 during later freeze-drying (Table 11). Experiments to determine whether Csp synthesis in MM160 was essential for lyophilization resistance were unsuccessful because erythromycin had a deleterious effect on cell recovery (Table 11).

Lactococcal activity after lyophilization. Growth curves from rehydrated lactococci suggested that HS and CS treatments reduced the time needed for cells to reach the midpoint of exponential growth (Fig. 3). Two-sample *t* test comparisons between means from untreated control cells and

TABLE 10. Influence of heat shock (HS) on the resistance of *Lactococcus lactis* subsp. *cremoris* MM160 and *L. lactis* subsp. *lactis* MM210 and FG2 to lyophilization

Strain and treatment	Cell fraction recovered[a]		Adjusted survival[b]	*P*	
	After HS	After FD		After FD[c]	Adjusted[d]
L. lactis subsp. *cremoris* MM160:					
None (control)	----	0.24 ± 0.06	----		
HS	1.00 ± 0.03	0.33 ± 0.03	----	< 0.10	----
HS + Ery[d]	0.78 ± 0.06	0.09 ± 0.01	0.11 ± 0.02	ND[f]	ND[f]
L. lactis subsp. *lactis* MM210:					
None (control)	----	0.20 ± 0.05	----		
HS	1.03 ± 0.08	0.48 ± 0.08	0.48 ± 0.11	< 0.02	< 0.05
HS + Ery[d]	0.93 ± 0.08	0.15 ± 0.04	0.16 ± 0.04	> 0.15	> 0.20
L. lactis subsp. *lactis* FG2:					
None (control)	----	0.25 ± 0.05	----		
HS	0.96 ± 0.03	0.63 ± 0.12	0.66 ± 0.11	< 0.02	< 0.02
HS + Ery[d]	0.95 ± 0.02	0.23 ± 0.03	0.24 ± 0.04	> 0.25	> 0.25

TABLE 10. (continued)

[a]Values represent the mean fraction (± SE) of the initial cell population recovered after HS for 25 min at 39°C (MM160) or 42°C (MM210 and FG2) and after freeze-drying (FD).
[b]Fraction of the post-HS cell population that was recovered after FD.
[c]Probability values were calculated from a two-sample *t* test comparison between the means from control and HS samples after FD.
[d]Probability values were calculated from a two-sample *t* test comparison between the means from control and the adjusted survival for HS samples.
[e]HS was performed in broth that contained 5 μg per ml of erythromycin.
[f]Not determined.

TABLE 11. Influence of 10°C cold shock (CS) on the resistance of *Lactococcus lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* to lyophilization

Strain and treatment	Cell fraction recovered[a]		Adjusted survival[b]	*P*	
	After CS	After FD		After FD[c]	Adjusted[d]
L. lactis subsp. *cremoris* MM160:					
None (control)	----	0.23 ± 0.005	----		
2 h CS	0.95 ± 0.10	0.34 ± 0.04	0.36 ± 0.06	< 0.025	< 0.05
2 h CS + Ery[d]	0.94 ± 0.08	0.18 ± 0.02	0.19 ± 0.01	< 0.05	< 0.025
L. lactis subsp. *cremoris* MM310:					
None (control)	----	0.23 ± 0.06	----		
2 h CS	1.01 ± 0.03	0.49 ± 0.02	0.48 ± 0.02	< 0.01	< 0.01
2 h CS + Ery[d]	0.98 ± 0.008	0.17 ± 0.11	0.18 ± 0.11	> 0.25	> 0.25
L. lactis subsp. *lactis* MM210:					
None (control)	----	0.23 ± 0.10	----		
2 h CS	0.96 ± 0.13	0.35 ± 0.07	0.37 ± 0.14	< 0.05	< 0.10
2 h CS + Ery[d]	0.94 ± 0.12	0.17 ± 0.13	0.19 ± 0.15	> 0.25	> 0.25

TABLE 11. (continued)

Strain and treatment	Cell fraction recovered[a]		Adjusted survival[b]	*P*	
	After CS	After FD		After FD[c]	Adjusted[d]
L. lactis subsp. *lactis* FG2:					
None (control)	----	0.25 ± 0.05	----		
2 h CS	0.94 ± 0.04	0.24 ± 0.03	0.25 ± 0.04	> 0.25	> 0.25
2 h CS + Ery[e]	0.89 ± 0.12	0.22 ± 0.06	0.26 ± 0.10	ND[f]	ND[f]

[a]Values represent the mean fraction (± SE) of the initial cell population recovered after CS and after freeze-drying (FD).
[b]Fraction of the post-CS cell population that was recovered after FD.
[c]Probability values were calculated from a two-sample *t* test comparison between the means from control and CS samples after FD.
[d]Probability values were calculated from a two-sample *t* test comparison between the means from control and the adjusted survival for CS samples.
[e]CS was performed in broth that contained 5 μg per ml of erythromycin.
[f]Not determined.

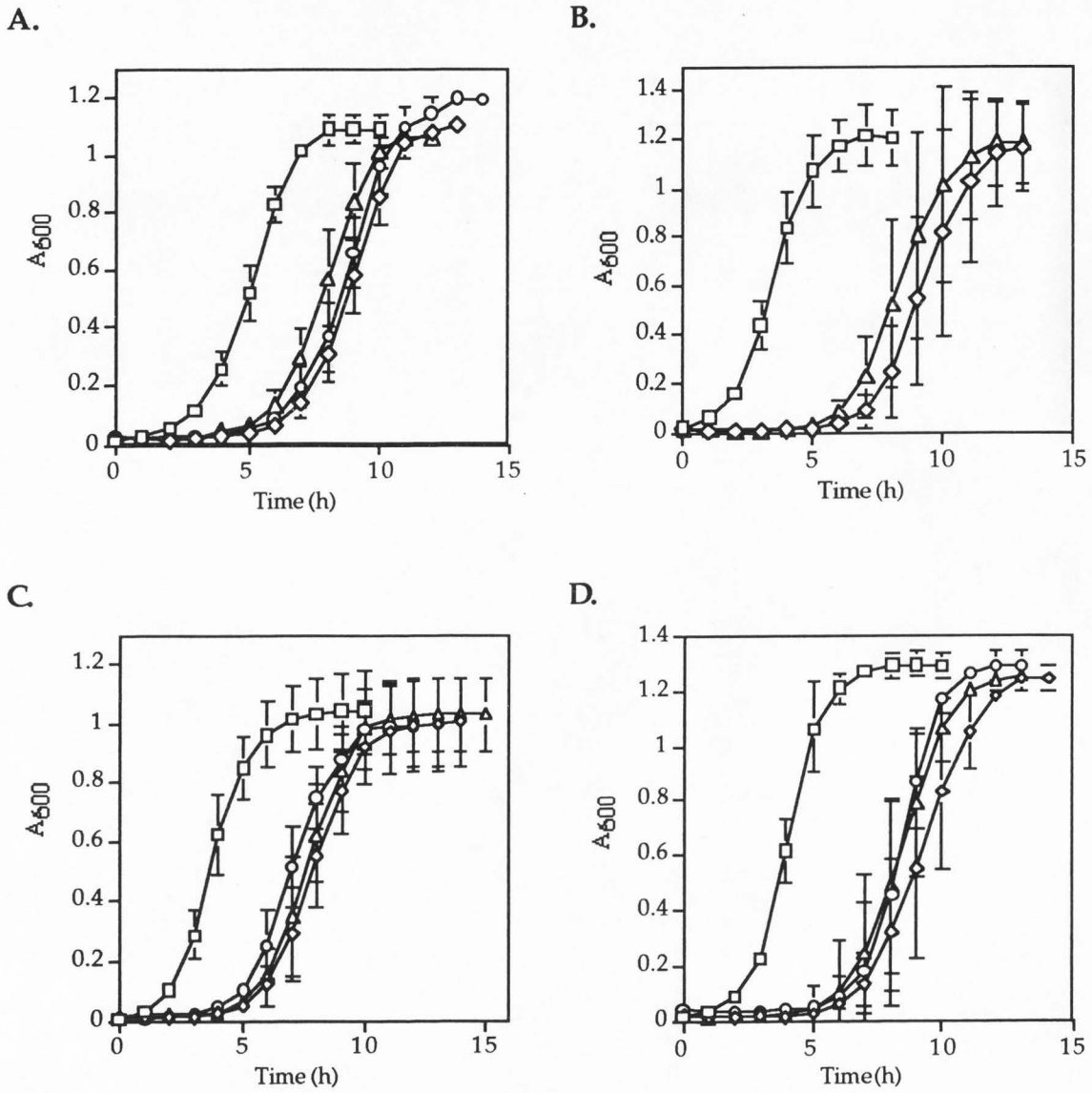


FIG. 3. Growth of lyophilized *Lactococcus lactis* subsp. *cremoris* MM160 (A) and MM310 (B) and *L. lactis* subsp. *lactis* MM210 (C) and FG2 (D) after rehydration. Symbols represent growth curves produced by a fresh overnight culture (□) and by lyophilized heat-shocked (○), cold-shocked (△), and untreated control (◇) cells after rehydration.

heat- or cold-shocked cell preparations confirmed that CS (but not HS) significantly ($P < 0.05$) reduced the time needed for *L. lactis* subsp. *cremoris* MM160 and MM310 to reach mid exponential growth, and that HS and CS reduced the time ($P < 0.05$) required by *L. lactis* subsp. *lactis* MM210 to attain the midpoint of exponential growth.

Similar experiments were performed to investigate the acid-producing ability of rehydrated lactococci. Once again, stress treatments appeared to reduce the time required for lyophilized *L. lactis* subsp. *cremoris* MM160 and *L. lactis* subsp. *lactis* MM210 to reach the midpoint on the b* color curve (b^*_{mid}) (Fig 4). Two-sample *t* test comparisons between means from untreated (control) cells and stress-treated cells showed that HS or CS treatment significantly reduced the time required by MM160 and MM210 to reach b^*_{mid} ($P < 0.0005$ or 0.0025, and $P < 0.01$ or 0.025, respectively). Finally, it may be noteworthy to mention that final b* values were higher in stress-treated versus fresh cultures for some strains (Fig 4C and 4D). The basis for this observation is unknown, but it may reflect HS- or CS-induced cross protection against acid stress in these bacteria.

Lactococcal cell membrane lipid composition. The cytoplasmic membrane is one of the primary targets for freeze- or desiccation-induced damage in cells (14, 55). For this reason, maintenance of membrane integrity is a central mechanism in desiccation tolerance in prokaryotes (55), and most bacteria maintain an ideal membrane state by altering the fatty acid composition (1). As growth temperature decreases, for example, cells typically

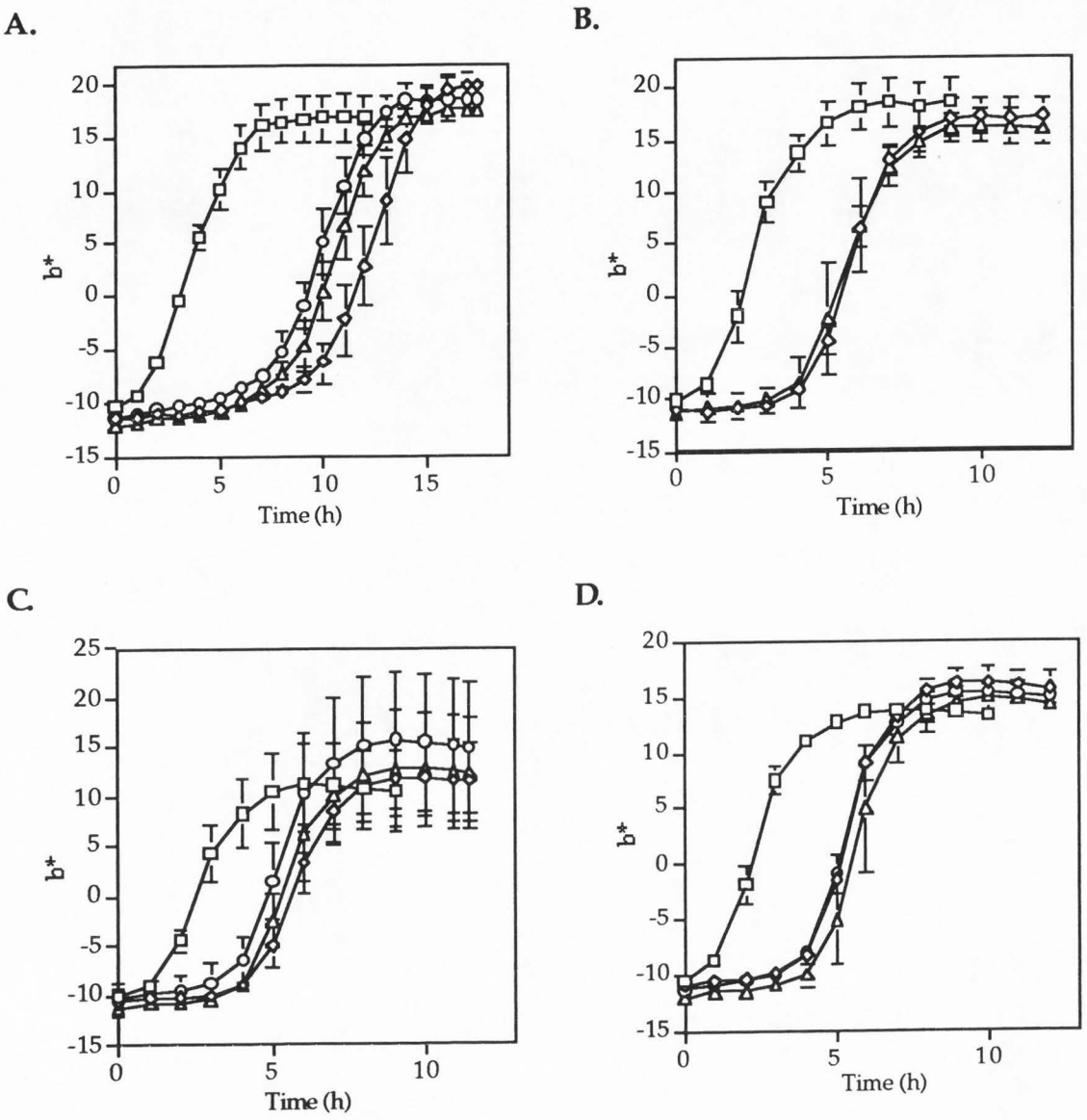


FIG. 4. Acidification of bromcresol-purple acid indicator medium by *Lactococcus lactis* subsp. *cremoris* MM160 (A) and MM310 (B) and *L. lactis* subsp. *lactis* MM210 (C) and FG2 (D). Symbols represent the change in b* (blue to yellow color) produced by a fresh overnight culture (□) and by lyophilized heat-shocked (○), cold-shocked (△), and untreated control (◇) cells after rehydration.

incorporate a higher percentage of lower-melting point (e.g., unsaturated) fatty acids into the cytoplasmic membrane to promote membrane fluidity, integrity, and function (1, 9).

Analysis of membrane lipid composition in stress-treated lactococci in this study indicated that membrane lipid alteration may also be a component of stress adaptation (Table 12). The membranes of heat-shocked MM160, MM310, MM210 and FG2 cells, for example, contained 19:0 cyclopropane fatty acid levels that were approximately 48, 35, 29, and 75% higher than untreated control cells (Table 12). The changes in membrane 19:0 cyclopropane acid content were not statistically significant, but this finding may be due to the fact that membrane fatty acid analyses were only performed in duplicate (and thus had only one degree of freedom in statistical analysis).

Membranes of cold-shocked lactococci did not contain an elevated concentration of cyclopropane, but these cells did contain a much lower ratio of saturated to unsaturated fatty acids (Table 13). Two-sample *t* test comparisons between means from untreated (control) cells and CS-treated cells confirmed that the ratio of saturated to unsaturated fatty acids in the cell membrane was significantly lower in cold-shocked MM310, MM210 and FG2 cells ($P < 0.05$, 0.05, and 0.01, respectively). Finally, linear regression analysis was performed to further characterize the relationship between membrane lipid changes and cell culturability after freeze challenge. Those data revealed that the correlations (r) between cell culturability after freeze challenge and the percent composition of 19:0 cyclopropane acid or the ratio

TABLE 12. Membrane lipid composition of *Lactococcus lactis* cells after heat shock (HS) or cold shock (CS) treatment[a]

Strain	Fatty acid	Treatment		
		None (control)	HS	CS
L. lactis subsp. *cremoris*:				
MM160	10:0	2.35 ± 1.32	1.90 ± 1.32	2.40 ± 1.15
	14:0	6.80 ± 0.68	7.10 ± 0.29	6.09 ± 1.34
	16:1	2.46 ± 3.47	2.17 ± 3.06	5.53 ± 1.16
	16:0	32.62 ± 1.79	32.94 ± 0.63	29.19 ± 0.09
	18:1	39.67 ± 9.18	32.10 ± 9.46	39.24 ± 8.98
	19:0	16.10 ± 10.22	23.79 ± 12.86	17.55 ± 9.23
MM310	10:0	2.69 ± 0.92	1.10 ± 0.41	1.45 ± 2.05
	14:0	9.62 ± 0.17	9.94 ± 0.45	8.64 ± 0.05
	16:1	4.70 ± 0.62	3.85 ± 0.16	6.39 ± 0.35
	16:0	40.07 ± 0.26	41.85 ± 1.02	35.10 ± 0.83
	18:1	27.58 ± 1.73	22.49 ± 0.21	35.36 ± 2.21
	19:0	15.34 ± 1.52	20.77 ± 0.53	13.06 ± 0.91
L. lactis subsp. *lactis*:				
MM210	14:0	6.24 ± 0.98	6.86 ± 0.69	5.84 ± 1.38
	16:1	1.40 ± 1.97	3.56 ± 1.45	4.83 ± 2.52
	16:0	34.43 ± 0.88	34.18 ± 1.87	30.73 ± 0.57
	18:1	36.95 ± 13.76	28.39 ± 16.77	34.90 ± 9.66
	19:0	20.98 ± 11.89	27.01 ± 17.04	23.70 ± 14.0

TABLE 12. (Cont)

Strain	Fatty acid	Treatment None (control)	HS	CS
L. lactis subsp. *lactis*:				
FG2	14:0	10.67 ± 0.58	11.18 ± 0.58	8.95 ± 0.33
	16:1	4.97 ± 0.91	2.24 ± 3.16	6.00 ± 0.19
	16:0	40.46 ± 0.27	42.71 ± 0.86	34.26 ± 0.96
	18:1	31.70 ± 0.67	22.51 ± 0.29	40.00 ± 0.26
	19:0	12.20 ± 0.54	21.36 ± 1.42	10.79 ± 0.70

[a]Values depict the percent of the total membrane lipid content (± SE) represented by each lipid species.

TABLE 13. Ratio of saturated to unsaturated fatty acids in the cell membrane of heat-shocked (HS) or cold-shocked (CS) *Lactococcus lactis*[a]

Strain	Treatment None (control)	HS	CS
L. lactis subsp. *cremoris*:			
MM160	0.72 ± 0.07	0.72 ± 0.05	0.60 ± 0
MM310	1.10 ± 0.04	1.12 ± 0.01	0.82 ± 0.04
L. lactis subsp. *lactis*:			
MM210	0.69 ± 0.01	0.70 ± 0.03	0.58 ± 0.02
FG2	1.05 ± 0.01	1.17 ± 0.07	0.76 ± 0.01

[a]Values represent the mean (± SE) from replicate analyses.

of saturated to unsaturated fatty acids in the cell membrane were 0.670 and 0.674, respectively (Fig. 5).

Influence of HS and CS on protein synthesis. As is shown in Fig. 6A and Table 14, autoradiography of 2D-PAGE gels that contained ^{35}S-labeled proteins isolated from *L. lactis* subsp. *cremoris* MM160 showed that HS at 39°C in MEB-Lc induced or increased the expression of at least 12 proteins. Similar experiments with *L. lactis* subsp. *lactis* MM210 revealed HS at 42°C in MEB-Ll triggered the expression of at least 9 Hsps (Fig. 6B and Table 14).

As shown by the arrows in Figs. 7 and 8, CS also resulted in the preferential expression of several proteins during the first 30 min of CS in MM160 and MM210. The MM160 Csps included 8 molecules while 15 Csps were detected in MM210 by CS treatment. Time course experiments showed that the ability to express other proteins was slowly restored over time, but Csp synthesis still remained a major component of total protein synthesis in both lactococci after 4 h at 10°C (Figs. 7 and 8).

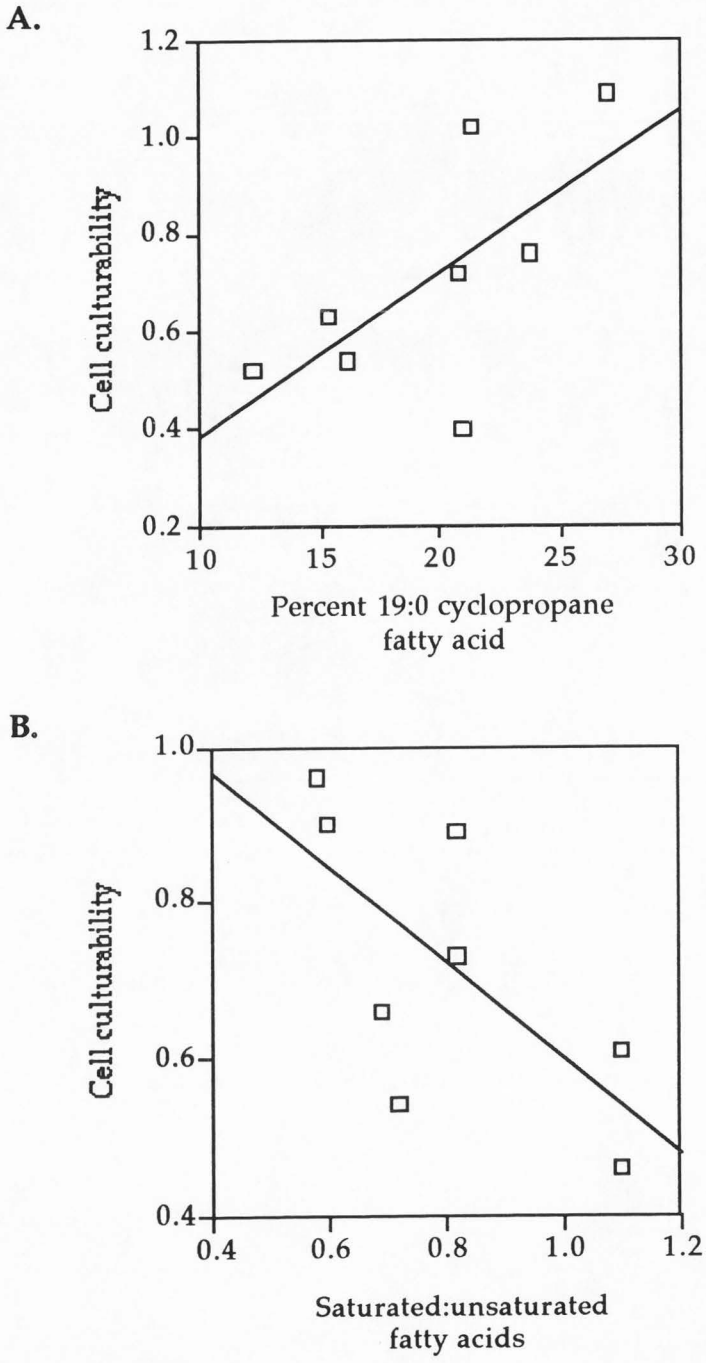


FIG. 5. Linear regression analysis of lactococcal cell culturability after freezing and (A) the percent composition of 19:0 cyclopropane fatty acid or (B) the ratio of saturated to unsaturated fatty acids in the cell membrane.

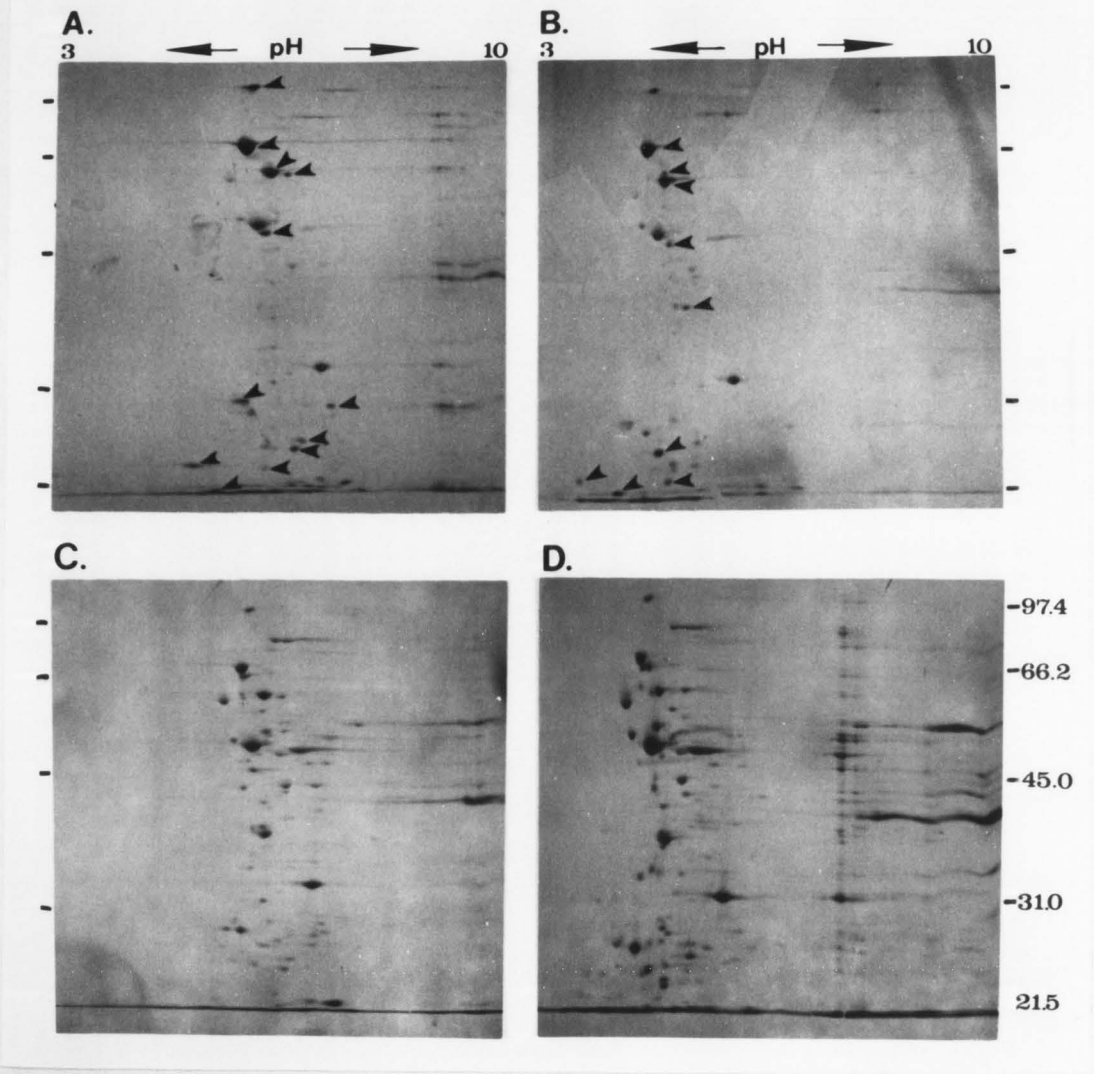


FIG. 6. Two-dimensional PAGE of [^{35}S]-labeled proteins isolated from heat-shocked *Lactococcus lactis* subsp. *cremoris* MM160 and *L. lactis* subsp. *lactis* MM210. Upper panels display proteins produced by MM160 (A) and MM210 (B) during HS for 25 min at 39 or 42°C, respectively. Lower panels show proteins synthesized by control MM160 (C) and MM210 (D) cells incubated at 30°C. Arrows identify heat shock proteins, and mass values for protein size standards are in kDa.

TABLE 14. Heat and cold shock proteins detected in *Lactococcus lactis* subsp. *cremoris* MM160 and *L. lactis* subsp. *lactis* MM210[a]

Heat shock proteins		Cold shock proteins	
MM160	MM210	MM160	MM210
94,000	----	70,300[b]	73,600[b]
75,100[b]	72,000[b]	---	70,200
----	66,800	62,700[c]	64,700[c]
68,400[c]	64,500[c]	61,600	61,300
67,600	----	----	55,800
54,000	51,900	---	53,900
----	41,400	---	53,600
28,800	----	50,100	52,300
28,100	----	42,100	45,200
----	24,800	35,200	36,400
24,700	----	----	33,100
24,100	----	28,200	29,600
22,600	22,400	24,100	25,100
22,200	22,300	----	24,000
20,600	21,500	----	23,600

[a]MM160 and MM210 heat or cold shock proteins with a similar mass and isoelectric position are placed on the same row. Mass estimates are in kDa.
[b]Putative DnaK homolog based on mass and isoelectric position.
[c]Putative GroEL homolog based on mass and isoelectric position.

FIG. 7. Two-dimensional PAGE of [^{35}S]-labeled proteins isolated from cold-shocked *Lactococcus lactis* subsp. *cremoris* MM160. Panels depict proteins produced by MM160 cells 10-30 min (A), 40-60 min (B), 100-120 min (C), 160-180 min (D), and 220-240 min (E) after temperature downshift from 30 to 10°C. Panel F shows proteins synthesized by control cells incubated for 20 min at 30°C. Arrows in panel A identify cold shock proteins and mass values for protein size standards are in kDa.

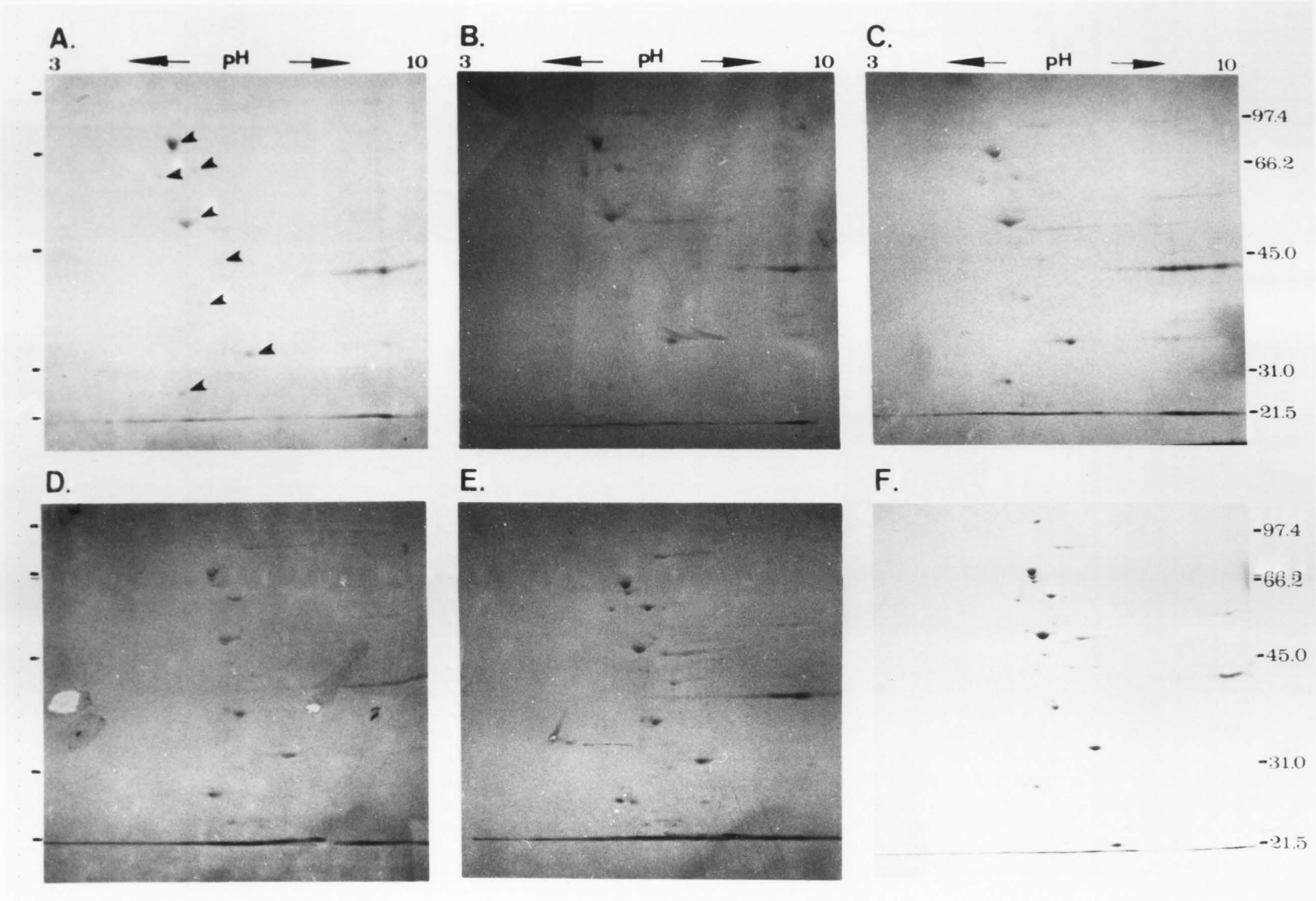
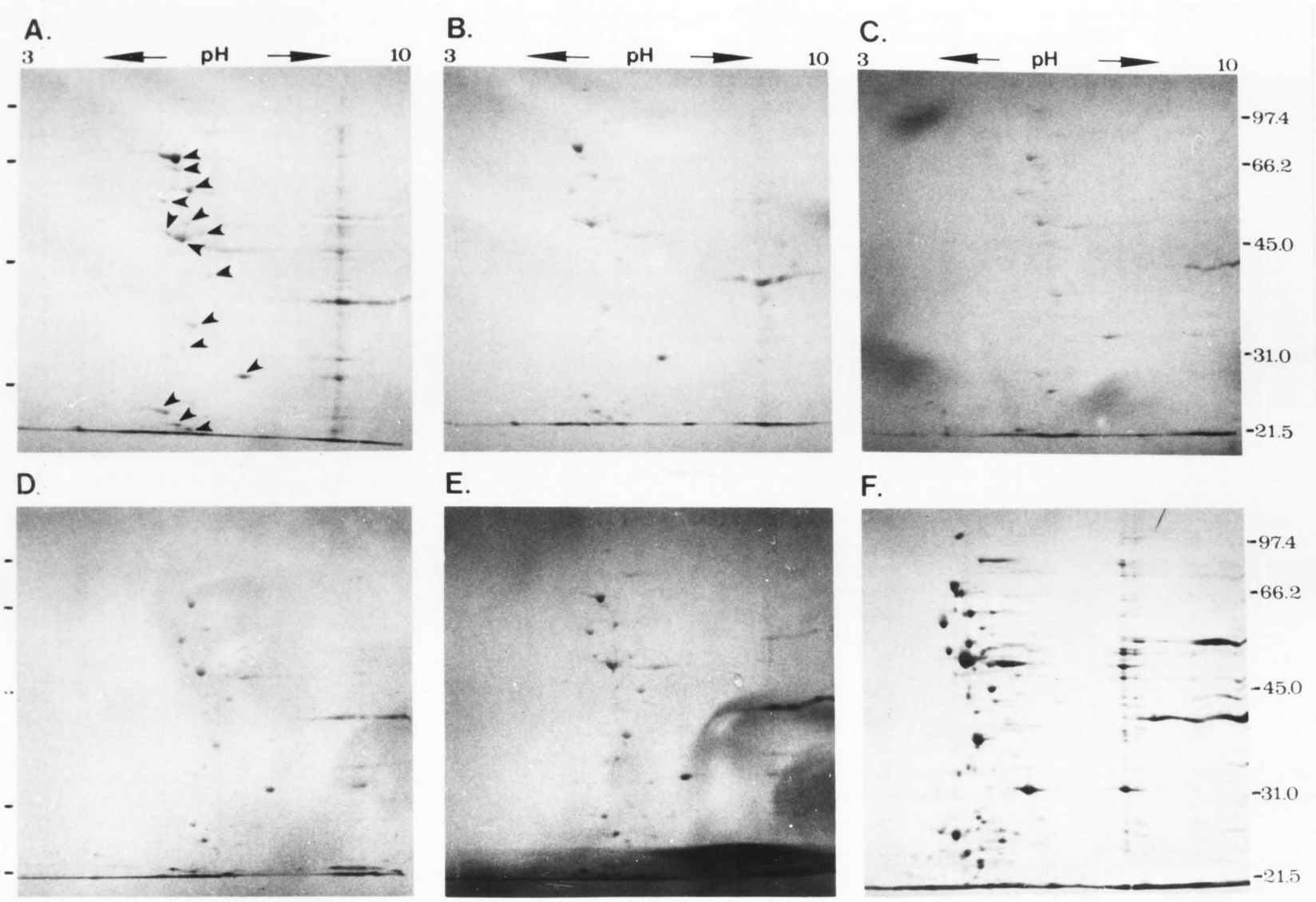

A.
3
pH
10
B.
3
pH
10
C.
3
pH
10
-97.4
-66.2
-45.0
-31.0
-21.5
D.
E.
F.
-97.4
-66.2
-45.0
-31.0
-21.5

FIG. 8. Two-dimensional PAGE of [^{35}S]-labeled proteins isolated from cold-shocked *Lactococcus lactis* subsp. *lactis* MM210. Panels depict proteins produced by MM210 cells 10-30 min (A), 40-60 min (B), 100-120 min (C), 160-180 min (D), and 220-240 min (E) after temperature downshift from 30 to 10°C. Panel F shows proteins synthesized by control cells incubated for 20 min at 30°C. Arrows in panel A identify cold shock proteins and mass values for protein size standards are in kDa.


A.
3
pH
10
B.
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pH
10
C.
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pH
10
97.4
66.2
45.0
31.0
21.5
D.
E.
F.
97.4
66.2
45.0
31.0
21.5

DISCUSSION

In industry, bacteria for lyophilization are collected in late exponential or early stationary phase, then frozen rapidly at -60 to -70°C (8, 59). These conditions are used because cells in the late exponential or early stationary phase of growth are typically most resistant to environmental extremes (20, 59), and rapid freezing at low temperature produces smaller ice crystals, which are less damaging to cells (24, 59). Although most lyophilized starter preparations are suitable for the inoculation of bulk starter tanks, expanding interest in ready-to-use culture concentrates for the direct inoculation of cheese vats has placed greater emphasis on lyophilized starter preparations that retain very high cell viability and activity. These requirements present a formidable challenge to the dairy industry because *L. lactis* subsp. *cremoris*, which are preferred over *L. lactis* subsp. *lactis* for cheesemaking (36), do not withstand environmental stress as well as the latter subspecies (8). For this reason, starter industry efforts to capitalize on lyophilization technology require an improved understanding of parameters which improve the survival and activity of starter bacteria during lyophilization (8, 29).

Previous work has shown that microbial adaptation to adverse conditions is frequently enhanced by the expression of an adaptive stress response (for reviews see 7, 11, 17, 19, 60). Since freezing and lyophilization each remove free water that is needed for biochemical reactions in cells, it was the hypothesis of this study that stress treatments which could enhance

lactococcal freeze tolerance would also help to promote cell viability during lyophilization. To test this hypothesis, it was first necessary to characterize the effect of HS, CS, and OS on cryotolerance in *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* starter cultures. These experiments were performed using mid-log phase cells because lactococci at this growth stage are quite susceptible to environmental stress (20) and are therefore likely to yield basic information on the effect of stress adaptation in cryotolerance.

Surprisingly, experiments to characterize the effect of stress treatments on lactococcal cryotolerance also required the identification of a growth medium in which control (untreated) cells did not withstand freezing for 24 h at -60°C very well. This temperature was selected for freeze challenge experiments because commercial-scale lyophilization of bacteria is typically initiated by freezing at -60°C to -70°C (59). Kim and Dunn (31) reported that the survival of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* cells grown and frozen in M17 ranged from 1- to 50% after 24 h at -20°C. When *L. lactis* subsp. *cremoris* MM160 and *L. lactis* subsp. *lactis* MM210 cells were grown and frozen in M17 broth, however, the fraction of cells that remained culturable after 24 h at -60°C was always greater than 0.87. This discrepancy may be due to the fact that the lower freeze temperature used in this study would produce more rapid freezing and smaller ice crystal formation, which are less damaging to cells (24). It is for this reason that commercial lyophilization of bacteria is initiated by rapid freezing at -60 to -70°C (59).

Efforts to identify a suitable growth medium in which control cells would not display high culturability under the freeze challenge conditions used in this study led to the selection of MEB-Lc and MEB-Ll broth media for strains of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis*, respectively. Freeze tolerance studies with lactococci grown and frozen in MEB-Lc or MEB-Ll showed that freeze resistance was variable among strains, but could be significantly improved ($P < 0.05$) in most strains by HS or CS treatment (Tables 3, 4, and 6). Phase contrast microscopy of control, HS-, and CS-treated lactococci showed increased cell recovery after freezing was not an artifact of stress-induced bacterial chain disruption (Table 15, appendix). In addition, increased freeze resistance was not detected when stress treatments were performed in broth that contained erythromycin (Tables 3, 4, and 6), which indicated stress-inducible proteins were required for cell protection.

Our observation that HS improved cryotolerance in *L. lactis* was in agreement with the data of Komatsu et al. (32), who showed that HS significantly improved the viability of *Saccharomyces cerevisiae* during freezing and thawing. In contrast, Panoff et al. (52) found that HS at 42°C for 30 min did not enhance the ability of *L. lactis* subsp. *lactis* IL1403 to survive repeated freeze-thaw cycles. The discrepancy between this study and the work of Panoff et al. (52) may be due to the differences in freeze-challenge conditions used in each study, or it may reflect the natural variation that occurs among strains of *L. lactis*. The latter possibility is based on the fact that Panoff et al. (52) focused their work on a single strain, *L. lactis* subsp. *lactis*

IL1403, and our data show that HS does not improve cryotolerance in all *L. lactis* strains (Table 5).

Cold shock-induced cryotolerance has been reported in many bacteria, including *L. lactis* (17, 31, 52). Kim and Dunn (31) recently reported that a 2-h CS at 10°C increased the cryotolerance of *L. lactis* subsp. *lactis*, but not *L. lactis* subsp. *cremoris*. In this study, CS at 10°C for 2-3 h significantly improved cryotolerance in all *L. lactis* subsp. *cremoris* and L. *lactis* subsp. *lactis* strains tested (Table 6). The basis for this discrepancy is unclear, but it may be due to differences in CS or freeze-challenge conditions used in each study. Although Kim and Dunn (31) did not discuss the rationale used to select the 2-h CS treatment, analysis of lactococcal culturability before and after CS treatment in this study revealed that prolonged (3 to 4 h) CS usually had a deleterious effect on cell viability (see Table 6; cell fraction recovered after CS). This effect was not due to a decrease in the ability of CS to protect cells because two-sample *t* tests between the means from control and adjusted survival (i.e., the fraction of the post-CS population recovered after FC) showed that cryotolerance in the post-CS population typically increased with CS time (see Results). Thus, the decline in lactococcal cryotolerance noted between 2- and 4-h CS treatments was a reflection of the cell fraction that was lost during CS, and the 2-h CS treatment represented the experimental point at which maximum cell protection was balanced against minimum cell loss.

Osmotic shock is another stress response that may help cells survive in low-moisture environments (10). In many bacteria, exposure to a hypertonic

environment induces osmotic shock proteins that effect the intracellular accumulation of specific solutes, by transport from the growth medium or de novo synthesis, to concentrations that retard water efflux. Since the molecules involved in this process must not be inhibitory to the cell at high concentrations, they are termed compatible solutes (10, 38). Important compatible solutes in lactic acid bacteria include betaine, proline, and γ-aminobutyric acid (21, 42, 44). Many species of bacteria are able to synthesize proline and γ-aminobutyric acid, but betaine must usually be obtained from the growth medium (42, 44). This compound is a natural component of plant and animal material, however, and lactic acid bacteria are routinely grown on rich media that contain good sources (e.g., yeast- and beef extracts) of betaine and other compatible solutes (28, 44). A key role for compatible solutes in the protection of lactic acid bacteria against drying was recently illustrated by Kets et al. (29), who showed that sublethal salt stress induced betaine accumulation and desiccation tolerance in *Lactobacillus plantarum, Lactobacillus halotolerans,* and *Enterococcus faecium.* Since desiccation tolerance was not detected in a fourth species (*Lactobacillus bulgaricus*) that was unable to accumulate betaine, those authors concluded that resistance to drying was directly related to the ability to accumulate compatible solutes (29).

Previous work by Molenaar et al. (44) showed *L. lactis* subsp. *lactis* ML3 contained high levels of betaine or proline when it was grown in a hyperosmotic medium, and because freezing and drying each subject cells to a low water stress (24), it was our hypothesis that OS may enhance the

resistance of *L. lactis* to freezing. As is shown in Tables 8 and 9, however, OS treatment improved cryotolerance in only one strain, *L. lactis* subsp. *lactis* MM210. One explanation for our result is that the media used in this study, MEB-Lc and MEB-Ll, did not contain enough betaine to satisfy the osmoregulatory needs of most *L. lactis* strains. This possibility seems unlikely, however, because yeast extract is the primary source of betaine in growth media for lactic acid bacteria (28), and the MEB-Lc medium used for OS in *L. lactis* subsp. *cremoris* strains contained twice as much yeast extract as the MEB-Ll medium used for MM210 (Table 1). Another explanation is that the salt sensitivity displayed by *L. lactis* subsp. *cremoris* (Table 7) may, as was noted by Kets et al. (29) for *Lactobacillus bulgaricus*, reflect an inability by these bacteria to accumulate compatible solutes. Since intracellular levels of betaine were not followed, however, we cannot make any clear conclusions regarding the role of compatible solutes in freeze protection of *L. lactis*. Nonetheless, because our data showed OS treatments did not induce cryoprotection in at least two strains of *L. lactis*, this treatment was excluded from our subsequent studies of lactococcal survival during lyophilization.

It was the hypothesis of this study that stress treatments which enhanced lactococcal freeze tolerance would also have a protective effect against lyophilization. As is shown in Tables 3 and 10, the HS treatment that improved freeze resistance in *L. lactis* subsp. *lactis* MM210 and FG2 also produced a significant increase in the culturability of these strains after lyophilization ($P < 0.05$). Similar results were obtained with CS-treated *L.*

lactis subsp. *cremoris* MM160, MM310, and *L. lactis* subsp. *lactis* MM210 (Tables 6 and 11). Improvements in lactococcal culturability after lyophilization were not detected when stress treatments were performed in broth that contained erythromycin, which indicated that Hsp and Csp expression were essential for this effect. Interestingly, these data suggest that HS induced the most significant increase in the resistance of *L. lactis* subsp. *lactis* strains against lyophilization, while CS treatments were more effective for strains of *L. lactis* subsp. *cremoris*.

As is shown in Fig. 3, HS and CS treatments also appeared to reduce the time needed for rehydrated lactococci to reach the midpoint of exponential growth, but two-sample *t* test comparisons showed this effect was only significant ($P < 0.05$) in cold-shocked *L. lactis* subsp. *cremoris* MM160 and MM310 and cold- or heat-shocked *L. lactis* subsp. *lactis* MM210. Nonetheless, experiments to monitor acid production by rehydrated cells showed HS and CS treatments significantly reduced the time required by MM160 and MM210 cells to reach b^*_{mid} (Fig. 4A and 4C).

Not surprisingly, bacteria from a fresh overnight culture always had a shorter lag phase than did freeze-dried cells (Fig. 3 and 4). Since freezing and desiccation will damage proteins, nucleic acids, membranes, and other macromolecules in bacteria (55, 59), the elongated lag phase noted in freeze-dried cells probably reflects the time required for cellular repair processes to restore the basic machinery needed to support growth.

In an effort to identify some of the physiological changes that may promote freeze and lyophilization resistance, we investigated the cell membrane lipid composition and stress protein expression in heat- or cold-shocked lactococci. The cytoplasmic membrane is one of the primary targets for freeze- or desiccation-induced damage in cells, so the ability of bacteria to modify membrane fatty acid composition has important practical consequences on their resistance to low-moisture environments (14, 55). Data presented in Tables 12 and 13 showed that heat-shocked cells had a higher 19:0 cyclopropane acid content than control cells while cold-shocked cells contained a lower ratio of saturated to unsaturated fatty acids. These changes are significant because maintenance of membrane integrity is a central mechanism in desiccation tolerance among microorganisms (55), and an increased concentration of cyclopropane or unsaturated fatty acids in the lactococcal cell membrane would be expected to increase cell membrane fluidity and promote membrane functions at low temperature. Work by Goldberg and Eschar (16), for example, showed that growth of *L. lactis* in a medium that increased the ratio of unsaturated to saturated fatty acids in the cell membrane also improved the survival of these bacteria during freezing. As a whole, our membrane lipid data suggest stress-induced changes in cell membrane composition probably contribute to enhanced freeze and lyophilization resistance, but other factors must also be involved in cell protection because similar changes were detected in strains whose resistance to freezing and lyophilization was not improved by HS or CS. This

conclusion was supported by linear regression analyses which showed the correlation between lactococcal cell culturability after freezing and the percent composition of 19:0 cyclopropane fatty acid or the ratio of saturated to unsaturated fatty acids in the cell membrane was relatively weak (0.670 and 0.674, respectively) (Fig. 5).

Insight into physiological factor(s) that may improve the culturability of *L. lactis* during freezing or lyophilization was obtained from our studies of HS- and CS-induced protein synthesis. As shown in Tables 3 and 6, HS- or CS-inducible cell protection was never detected when stress treatments were performed in the presence of erythromycin, which indicated that de novo protein synthesis was required for this effect. Studies of de novo protein synthesis during HS or CS in *L. lactis* and other bacteria have shown that it is dominated by the transient induction of specific proteins, termed Hsps and Csps, respectively (17, 19, 51, 66). In this study, 2D-PAGE of ^{35}S-labeled proteins showed HS induced at least 9 and 12 Hsps in *L. lactis* subsp. *lactis* MM210 and *L. lactis* subsp. *cremoris* MM160, respectively (Fig. 6). These data are in reasonable agreement with previous studies that showed *L. lactis* produced 13-17 Hsps immediately after temperature upshift from 30 to 42 or 43°C (30, 66). Characterization of the *L. lactis* CS response by 2D-PAGE with [^{35}S]-labeled proteins has not been reported, but results that showed CS induced preferential expression of 8-15 Csps (Figs. 6 and 7) were also in reasonable agreement with existing studies (51).

Our finding that Hsp synthesis was required for HS-induced cryoprotection in *L. lactis* subsp. *lactis* MM210 and FG2 (Table 3) was in agreement with previous work that showed HS significantly improved the viability of *Saccharomyces cerevisiae* during freezing and thawing, and that this effect depended on de novo Hsp synthesis (32). Those researchers later speculated that the cryoprotective effect of Hsps may be related to their function as macromolecular stabilizers (i.e., chaperones) (33). Macromolecular stabilizers can strengthen the hydrogen-bonded structure of water and, as a consequence, increase the level of unfreezable water that surrounds macromolecules (33). This action, which is also characteristic of some cryoprotective agents, can promote cell survival during freezing or drying because enzyme activity requires only a monolayer of water over the protein (33, 55).

In *L. lactis* and other bacteria, two Hsps that may function as macromolecular stabilizers during cell freezing or drying include the highly conserved chaperones Hsp70 (DnaK) and Hsp60 (GroEL) (66). DnaK, for example, has a crucial role in osmotic adaptation of *Escherichia coli*, and this activity is thought to involve stabilization of membrane proteins or other membrane-related functions (43, 68). Although identification of stress proteins was not an objective of this study, putative identification of DnaK and GroEL was performed using M_r values and the characteristic isoelectric position of these two *L. lactis* chaperones on 2D-PAGE (30). By this approach, we propose that the MM160 and MM210 HS-inducible proteins Hsp75.1 and

Hsp72, respectively, are DnaK, and that the MM160 Hsp68.4 and MM210 Hsp64.5 are GroEL (Table 14 and Fig. 6). More significantly, we also found that MM160 and MM210 Csps included molecules with M_rs and isoelectric positions on 2D-PAGE that were essentially identical to those of DnaK (Csp70.3 and Csp73.6, respectively) and GroEL (Csp62.7 and Csp64.7, respectively) (Table 14 and Figs. 7 and 8), and expression of these proteins was a major component of total protein synthesis for at least 4 h after temperature downshift from 30 to 10°C. DnaK is not a Csp in *E. coli* or *Bacillus subtilis* (18, 22), but recent work by Lelivelt and Kawula (37) demonstrated *E. coli* has an Hsp70 homolog (Hsc66) that is encoded by a cold-inducible gene, *hscA*. Those authors (37) also showed that the Csp profile was altered in an *hscA* null mutant, but they did address the effect of Hsc66 loss on cryotolerance. Osmotic shock- or CS-inducible expression of GroEL has not been reported in *E. coli* or *B. subtilis* (18, 22), but Kilstrup et al. (30) recently showed DnaK and GroEL were induced by OS in *L. lactis*. In addition, *L. lactis* Csps with the M_rs and isoelectric positions on 2D-PAGE of Hsp70 and Hsp60 were detected, but not identified, by Panoff et al. (51). As a whole, these data support the hypothesis of Komatsu et al. (33) that stress-induced chaperone proteins have cryoprotective effects that stem from their ability to increase the level of unfreezable water that surrounds macromolecules.

SUMMARY AND CONCLUSIONS

Lactococcus lactis subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* are important starter bacteria for the manufacture of cheese and fermented milks (34). Industrially, lactococci may be preserved or distributed as liquid, spray-dried, frozen, or lyophilized preparations (54, 61). These preparations are all suitable for the inoculation of bulk starter tanks, but expanding interest in ready-to-use culture concentrates for the direct inoculation of cheese vats has placed greater emphasis on starter production and preservation methods that promote high cell viability and activity. These requirements can present a formidable challenge because starter preservation and production methods typically subject bacteria to stress conditions such as freezing, drying, and concentration stress, which diminish cell vitality (40, 54). This limitation is compounded by the fact that *L. lactis* subsp. *cremoris*, which are preferred over *L. lactis* subsp. *lactis* for cheesemaking (36), do not withstand environmental stress as well as the latter subspecies (8).

Studies of microbial adaptation to environmental fluctuation have revealed that cell survival under adverse conditions is frequently enhanced by the expression of an adaptive stress response (for reviews see 7, 11, 17, 19, 60). Microbial resistance to freezing or drying, for example, may be enhanced by deliberate induction of a HS, CS, or OS response (29, 32, 67). Previous work has shown that *Lactococcus lactis* has active HS, CS, and OS responses (44, 51, 66), and this study investigated their effect on cryotolerance in the

commercial starter bacteria *L. lactis* subsp. *cremoris* MM160 and MM310 and *L. lactis* subsp. *lactis* MM210 and FG2. The ability to withstand freezing at -60°C for 24 h was variable among lactococci, but resistance to this treatment was significantly improved ($P < 0.05$) in most strains by a HS or CS treatment (Tables 3, 4, and 6).

Since freezing and lyophilization each remove free water that is necessary for biochemical reactions in cells, it was our hypothesis that stress treatments which could enhance lactococcal freeze tolerance would also help to promote cell viability during lyophilization. Data presented in Tables 10 and 11 confirmed that HS or CS treatments that improved lactococcal freeze resistance generally induced a significant improvement ($P < 0.05$) in cell culturability after lyophilization. One of the interesting observations to emerge from those data was that CS appeared to be the most effective method to increase lyophilization resistance in *L. lactis* subsp. *cremoris*, whereas HS treatments were more effective for *L. lactis* subsp. *lactis*. The basis for this trend is unknown, but the intimate role of Hsps and Csps in cell protection suggests that further study of stress protein function may facilitate the development of *L. lactis* starters that are constitutively resistant to freezing or lyophilization.

Our investigations of stress-induced physiological changes in *L. lactis* that may influence cell culturability during freezing or lyophilization indicated that Hsps and Csps may function in at least two ways. First, HS- and CS-inducible chaperone proteins such as DnaK and GroEL may function as

cryoprotective agents by virtue of their ability to increase the level of unfreezable water that surrounds macromolecules. Since enzyme activity requires only a monolayer of water over the protein (55), molecules that can increase the unfreezable water content of cells may be very important for cell survival during freezing or drying. In addition, Hsps and Csps may have roles in the regulation of cell membrane lipid content. As shown in Tables 12 and 13, heat-shocked cells had a higher concentration of 19:0 cyclopropane than control cells, and cold-shocked cells contained a lower ratio of saturated to unsaturated fatty acids. In conclusion, our data show that the resistance of commercial *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* cheese starter cultures to freezing and freeze-drying can be significantly improved by HS or CS treatment. This result suggests that an improved understanding of adaptive physiology in *L. lactis* would facilitate efforts to improve the consistency, quality, and functionality of lyophilized starter preparations.

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APPENDIX

TABLE 15. Influence of heat shock (HS) and cold shock (CS) on the mean chain length of *Lactococcus lactis* strains used in the study[a]

Strain	Treatment		
	None (control)	HS[b]	CS[c]
L. lactis subsp. *cremoris*			
MM160	2.4 ± 0.2	2.3 ± 0.6	2.6 ± 0.7
MM310	2.0 ± 0.2	2.0 ± 0.2	2.0 ± 0.2
L. lactis subsp. *lactis*			
MM210	2.2 ± 0.5	2.1 ± 0.2	2.1 ± 0.2
FG2	2.0 ± 0.2	2.2 ± 0.3	2.3 ± 0.2

[a]Values represent the mean number of cells in each chain (± SE) present in 6 different fields of view under phase contrast microscopy.
[b]25 min at 39°C (*L. lactis* subsp. *cremoris* MM160 and MM310) or 42°C (*L. lactis* subsp. *lactis* MM210 and FG2).
[c]2 h at 10°C.